In re the Application of:

Birgit HELM (EURO DPC)

Serial no: 09/133,766 (Continuation)

Filed: 25 November 1993

Title: Allergen/Inflammatory Testing & Diagnosis

Signed: Birgit Helm

Dated: 14/12/1999

DECLARATION UNDER 37 CFR 1.132

I, Birgit Helm, declare that my credentials are as listed in the attached curriculum vitae;

I understand that the above patent application, of which I am an inventor, has been filed following rejection of Claims 16-24 of the parent over Gilfillan et al, Cantor et al and Levi-Schaffer et al, and of Claim 17 of the parent over Wilson et al, for lack of inventive step. In order to show the differences between the subject matter of the cited documents and the subject matter of my application, I hereby submit the following discussion of the cited documents and the present invention;

The Examiner accepts the novelty of Claims on file, primarily by virtue of the absence of a sensitising agent (human IgE).

We herewith make of record that statements made herein in respect of the claims on file should not in any way be taken as an admission of non-patentability of the claims of the parent application, now deleted, regarding methods employing a sensitising agent or sensitised cells.

The basis of this Declaration

Applicants have previously submitted that the present application relates to the use of unidentified "virgin" substances the allergenicity of which is not known and which is determined without the addition of IgE. Applicants submit with this response further illustration of the different assays which can be performed with the above known tools and the implications thereof, specifically in the form of recent publications by the inventors which employ the method of the invention as the prime assay tool, as distinct from known methods as disclosed in Gilfillan et al, Cantor et al and Levi-Shaffer et al. The publications, which are filed after the priority date of this application serve merely as illustration, distinguishing the nature of the known and novel assay (determining potential allergens as opposed to studies with known allergens) and moreover illustrating the significance of that distinction. Applicants respectfully submit that the distinction is so significant in terms of the usefulness of the method of the invention that not even with hindsight could the teachings of Cantor et al, Gilfillan et al and Levi-Shaffer et al be considered to render it obvious.

The general state of the art

The prior art and the present invention relate to the technology in general of : using certain cell lines, which are cells of the innate and/or adaptive immune responses, preferentially cells of mast cell/basophil lineage, capable of synthesising and/or releasing cellular mediators mediators linked to the development of type I hypersensitivity = atopic or allergic responses.

The increase in allergic disease has been attributed to environmental factors, which are thought to enhance the synthesis of the allergic antibody immunoglobulin (Ig)E, although no unifying feature has emerged from numerous and extensive studies of many pro-allergenic substances. Consequently, there is no generally accepted means of predicting or assaying for the potential allergenicity of environmental agents. In mammals, allergens include a large number of seemingly diverse and mainly innocuous antigens, such as plant pollens, fungal spores, latex associated products, insect venoms, mite and cockroach emanations. (Reviewed in Helm 1994[1], Dudler et al. 1995[2], Machado et al. 1996[3] and Helm et al 1998[8]). In addition, there is evidence that pollutants in air such as diesel exhaust particles (Diaz-Sanchez, Tsieu, Fujieda[4]) or cigarette smoke (Smyth et al, 2000[5]) act as adjuvants to stimulate enhanced IgE synthesis.

An understanding of the molecular basis of potential allergenicity, i.e. the capability of an environmental substance to induce or enhance IgE synthesis provides the scientific rationale for the establishment of a biological assay system for the routine assessment of potential allergenicity and/or IgE adjuvant activity of aero-allergens and pollutants. This is analogous to the biological assay system for the screening of potential carcinogenicity, such as the 'Ames Test'.

The nature of and advantages provided by the present invention

We know that B-cell class switching to the synthesis of antibodies of the IgE isotype is critically dependent on the cytokines interleukin-(IL) 4 and IL-13. Therefore, stimulation of the synthesis of these cytokines by environmental antigens provides a molecular switch signal, which promotes IgE synthesis and the development of allergies and asthma. Any antigen or pollutant, which

induces the synthesis/secretion of these cytokines must be considered as potentially allergenic.

It is known that IL-4 and IL-13 are secreted from T-helper 2 cells and human and rodent cells of basophil/mast cell lineage in response to an IgE-mediated antigenic stimulus. In the latter this is observed after IgE synthesis has occurred.

Following the engineering of the RBL-2/2/C cell line (Wilson et al. 1994[6]), we observed IgE-independent mediator release, i.e. from RBL-2/2/C cells in control experiments. These cells responded with mediator secretion following exposure to components in bee venom (Helm 1994[7], Dudler et al 1995[2]), house dust mite emanations, proteases of fungal and parasitic origin, and diverse lectins and viral haemagluttinins (Machado et al. 1996[3], Helm et al. 1998[8]). Degranulation in response to exposure to these allergens without IgE was linked to the synthesis and secretion of the cytokine interleukin-4, which is known to be essential for the induction of IgE synthesis. Human mast cells and basophils showed similar cellular responses following exposure to these potential allergens providing evidence for the commonality of cellular responses between species. Furthermore, environmental pollutants, such as diesel exhaust particles (Diaz-Sanchez, 1997 Tsieu, 1998 Fujieda[4].) and cigarette smoke stimulate synthesis and secretion of pro-allergenic cytokines from human and rodent cells (Smyth et al[5] 2000).

These findings indicate that the reasons why diverse aero-allergens, insect venoms or invading parasites stimulate IgE synthesis, can be explained by their ability to induce synthesis and secretion of cellular mediators, including pro-inflammatory cytokines in non-sensitised cells. This is directly linked to the development of allergic responses as demonstrated by the studies of Dudler et al[2]. The prior art studies response in already sensitised systems.

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It is important to note that the cellular responses are reproducibly observed with cells of the human and rodent immune system in the absence of sensitisation with allergen specific IgE, i.e. at a point prior to the establishment of allergic responses.

The application therefore does not relate to the technology in general of: "binding human IgE as substituent for human $Fc_{\varepsilon}RI$ as a model or assay tool"; using the tool to test the efficacy of anti-allergic compounds by sensitising the cells, exposing to allergic compound following binding of IgE and release of mediators.

Furthermore, the applicants do not dispute that these techniques are established tools for working with sensitised cells and known allergens. This is the subject matter indeed of the cited prior art documents.

The current application relates to the employment of cells of mast cell/basophil lineage to the assessment of the capability of environmental allergens and pollutants to induce and/or enhance the synthesis/secretion of mediators which stimulate IgE synthesis and induce allergic responses. The disclosures of Gilfillan et al, Cantor et al, and Levi-Schaffer et al are therefore unrelated to the claims made in this patent. The novelty of our approach derives from the identification of secretor variants of the RBL cell line as a means of identifying pro-allergenic properties of environmental agents, using as a 'read-out" stimulation of synthesis secretion of pro-allergenic cytokines. At present, we cannot rule out that, in addition to mast cells and basophils, other cells, e.g. T-helper cells may be activated to produce pro-inflammatory cytokines by allergens and pollutants in the absence of sensitisation with IgE and prior to the establishment of adaptive immune responses.

The observation that many common allergens and pollutants can trigger pro-inflammatory cytokine secretion indicates that a bioassay can be employed to predict the allergenicity of defined and complex mixtures of environmental agents. The claims inherent in this patent refer to the use of any cell system capable of producing pro-inflammatory cytokines, exemplified by RBL cells, but for which we have additional evidence in respect of cloned T cells, epidermal cells from bronchae and the like, independent of their transfection status with e.g. FcεRIá (Wilson et al[6]) as a model system to assess the potential allergenicity. The observation that rodent and human mast cell lines exhibit similar responses provides the justification for the use of a rodent cell line to assess potential allergenicity to humans. This assay system is envisaged as a preliminary screening test, employing as a "readout" the induction of pro-inflammatory cytokine synthesis following exposure to a potential allergen/pollutant. The experiments described by Dudler et al[2]. indicate the usefulness and sensitivity of this methodology in differentiating between potential allergenicity, or lack of this activity, between e.g. homologous substances differing in e.g. one amino acid residue, which was confirmed in *in vivo* studies in a murine model system (Dudler et al 1995[2]).

The bio-assay we propose in this patent aims at the implementation of allergen test screens linked to avoidance strategies. This can be applied in a pro-active manner to prevent exposure before allergic sensitisation occurs. This is particularly important in the current situation where the list of compounds and sources and potentially inadvertent exposure, including that from genetically engineered plants, is likely to increase.

Examples of the application of the assay of the invention

We refer to BBSRC Business [9], and:

and human lung mast cells

Smyth et al[5], accepted for publication, illustrates the present invention in identifying pollutants in cigarette smoke which induce a mast cell response. In the examples RBL cells were exposed to cigarette smoke and mediator release determined. Nature and type of mediator release was observed and the results were used to analyse components in cigarette smoke responsible for potential immune modulatory activity, which can in turn be used to assist in elimination from tobacco or permit the assessment or development of potential antagonists. It is important that this study uses unsensitised cells since only by this means can the potential allergenicity of a substance be determined, and studied without distortion of results by sensitised response.

Helm et al[8] at 3.2 and particularly line 4 at page 203 the distinction of the invention is clearly stated and explained. "In order to assess the technology (of the invention) we sensitised the cells with the serum of a bee-venom sensitive individual (EMC) and, following challenge with major bee-venom allergen phospholipase A_2 (PLA_2), we could, as expected, demonstrate mediator release. Surprisingly, however, control experiments, where non-sensitised cells had been incubated with the same concentration of antigen in the absence of serum containing bee-venom specific IgE, cells also responded with the de-granulation of the cellular mediators". The paper goes on to state that this discovery enabled further studies to be carried out distinguishing active and inactive genetically engineered venom components. These observations were found to be applicable to a full range of occupational and environmental allergens (Helm et al 1998).

Respectful consideration is requested.

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Adv. Exp. Med. Biol. 347: 1-10

ref 7

IS THERE A LINK BETWEEN THE NATURE OF AGENTS THAT TRIGGER MAST CELLS AND THE INDUCTION OF IMMUNOGLOBULIN (IG)E SYNTHESIS?

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THE ALLERGIC RESPONSE

What is allergy? Usually the statement "*I am allergic*" suggests that one responds to the contact with a seemingly harmless extraneous entity in an adverse and exaggerated fashion. The term was originally coined by Pirquet in 1906, meaning "altered reactivity", it was used to characterise the change in reaction which has occurred following exposure to an antigen. Present day immunologists attribute the manifestations of allergy to a condition where the immune system replies to the initial encounter with a foreign substance by producing an antibody of the immunoglobulin (Ig)E isotype, and the role of IgE antibodies in mediating the clinical symptoms of both immediate and delayed hypersensitivity reactions has been extensively documented since their discovery as a separate Ig class in 1966 [1].

IgE antibodies are made up of two light chains (κ or λ isotype) which combine with two ε-chains to form the variable region involved in antigen recognition. The ε-chain is a five domain glycoprotein which forms a covalent homodimer via two cystine residues linking opposite Cε2 domains. In normal individuals, the plasma levels of this antibody are lower than those of any other class; their synthesis is thought to be tightly controlled by a network of B- and T-lymphocytes [2], which is probably a reflection of the involvement of this

Immunobiology of Proteins and Peptides VII
Edited by M.Z. Atassi, Plenum Press, New York, 1994

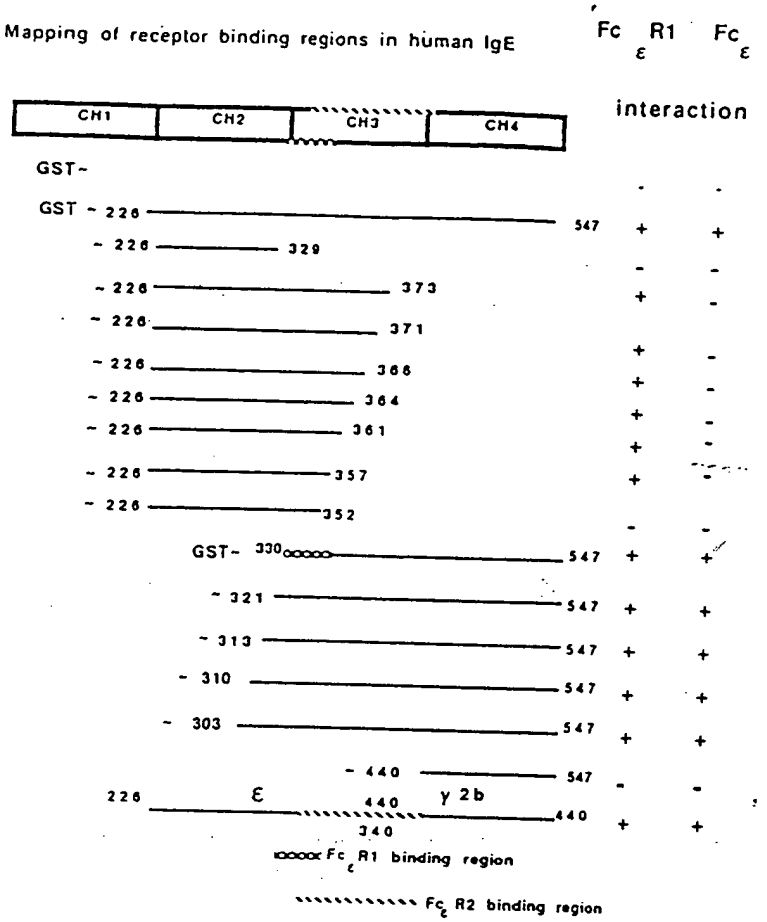

Figure 1. Recombinant ε-chain fragments were expressed in *E. coli* as gluthathione S-transferase fusion proteins and isolated by standard procedures. To measure rFcε/FcεR1 interaction, the transfected RBL-2H3 cell-line was incubated for 24h at 37°C with 10^{-6} M dexamethazone to induce receptor expression[18], after washing with buffer, cells were incubated with 200 fold molar excess rFcε peptides for 45 min at 20°C before addtion of ^{125}I-labelled hIgE V_{NP}. Cells were washed, lysed, and radioactivity was counted.

FcεR2/CD23 interaction was measured by FACScan of 8866 lymphoblastoid leukaemia cells bearing FcεR2[8].

antibody isotype in the activation of the release of cytotoxic mediators from effector cells that bear the class-specific Fc-receptors for this Ig [3]. IgE has not only the lowest levels but also the shortest serum half-life of all classes of immunoglobulins. The majority of IgE molecules are found bound to cells expressing high- (FcεR1) and low-affinity receptors (FcεR2) [4].

These receptors are present on leukocytes in the blood and the cells lining the mucosal membranes of the gut and the respiratory organs. Employing a series of overlapping ε-chain derived fragments shown in Figure 1 we found that the site(s) in IgE that interact with both receptors are located in the Cε3 domain [5], an observation also made by others [6,7]. The region(s) in IgE that interact with FcεR2 are close to, but not identical to the site(s) that engage the mast cell and basophil receptor [5,7,8]. There is evidence that IgE bends out of

plane at the interface between the Cε2 and Cε3 domain with both Fab arms and the C-terminal end of the Fc segment sticking out, away from the membrane [9].

Cross-linking by cognate antigen/allergen of cellular receptors occupied by IgE results in the release of the pharmacologically active agents that cause the clinical symptoms of allergy; but there is also compelling evidence that these mechanisms will confer protective immunity to parasites and some viral infections [3,10]. This beneficial effect is however generally considered to be of limited value in populations that live in an environment where parasitic infestations are rare, and the synthesis of an antibody of the IgE isotype is usually regarded as an unwanted immune response. Allergic reactions are rarely fatal, but with more than 20% of the human population being afflicted by some type of allergy and evidence that the incidence of the disorder is increasing world-wide, the development of effective therapeutic interventions in atopic disease is of considerable medical, commercial and social importance and an improved understanding of the molecular mechanisms involved in the genesis of the allergic response may expedite such a quest.

FACTORS INFLUENCING THE ANTIGEN/ALLERGEN SPECIFIC IMMUNE RESPONSE

At birth, IgE levels are not measurable or exceedingly low and the mechanisms that give rise to an IgE response in certain individuals as the result of the encounter with a particular antigen are at present unresolved and the subject of intense investigations in many laboratories. Several diverse factors seem to contribute towards the development of allergic disease:

1. Individual predisposition

When exposed to e.g. airborne antigens, about 20-30% of the population develop IgE antibodies. Individual variations in mucosal permeability, which may influence antigen presentation, may account for some of the observed differences in response to allergens. In addition, familial tendencies in the development of atopic disease have been observed, nearly 50% of individuals who have two allergic parents develop allergies, compared to 15% in the normal population, although no simple pattern of Mendelian inheritance could be established. There is evidence for MHC-gene control, and it has been suggested that a recessive allele determines high IgE levels, but other genes may also influence the trait, and linkage studies suggest that a gene present on chromosome 11 controls the development of atopic disease [11].

2. Mediator release by effector cells

The symptoms of immediate hypersentivity are caused by the secretion of preformed and newly synthesized mediators from mast cells and basophils, which express high-affinity IgE receptors. Mast cell-mediated cytotoxicity resembles natural cytotoxic activity since the

release of mast cell-derived chemotactic factor of anaphylaxis' (ECF-A), tumor necrosis (TNF) and related cytotoxic factors can be demonstrated from cells of basophil/mast cell lineage. The chemotactic effect of these agents for the recruitment of monocytes, neutrophils and eosinophils is well known and infiltration of e.g. lung tissue by these cells has been observed in asthmatic patients in the late phase [12]. In addition, eosinophil-mediated cytotoxicity is dependent on mast cell-mediators, and in tissues affected by immediate and delayed type hypersensitivity reactions, eosinophils, mast cells/basophils are frequently found in close association. Another relevant observation in this context is that IgE-activated eosinophils generate mediators such as eosinophil peroxidase and oxygen metabolites which can induce mast cell secretion through a non-immunological stimulus [13]. There is also compelling evidence that mediators released by mast cells and basophils induce the selective activation and expression of FcεR2 on inflammatory cell sub-populations [3] which are involved in the late phase allergic reaction. An initial inhibition of mast cell and basophil activation is therefore highly desirable in order to reduce the clinical symptoms associated with both immediate and delayed hypersentivity.

Empirical observations suggest that the manifestation of allergic disease is closely linked to the serum concentration of IgE. The precise nature and sequence of events that determine class switching is still unresolved. Isotype specific synthesis is known to be influenced by cytokines [14], and the role of cytokines produced by T-cells in the regulation of IgE synthesis by B-cells has been recognised for some time [15]. In the mouse[16], two types of helper T cells have been distinguished based on their pattern of cytokine production: TH_1 cells produce IL-2, interferon- (IFN)γ, IL-3, and granulocyte macrophage colony stimulating factor (GM-CSF), while TH_2 cells produce IL-3, GM-CSF, IL-4, IL-5, and IL-10. Of particular interest is IL-4 which is known to promote FcεR2 expression on inflammatory cells, it is essential for the induction of IgE transcription by B-cells, while other TH_2 cytokines (Il-5) augment IL-4 induced IgE production, or suppress the negative effect of IFN-γ on IgE synthesis (IL-10). It is important to note in this context that the profile of cytokines secreted from activated mast cells/basophils which include IL-3, IL-4, IL-5, IL-6 and GM-CSF, resembles that of TH_2 cells [17]. Mast/basophil exocytosis may therefore play an important role in the differentiation of TH lymphocytes and the regulation of IgE production by B-cells.

3. The nature of the antigen

The encounter with a number of antigens seems to give rise with preference to the synthesis of an IgE antibody, suggesting that the isotype response may be related to the nature of the antigen. Most allergens are (glyco)proteins in the molecular weight range between 5-75 kD. At present, it is not known why certain seemingly innocuous and diverse substances like pollen grains, mould spores, house dust mite or cockroach emanations,

animal dander or insect venoms, latex proteins or industrial pollutants elicit an allergic response in some subjects but not in others. Similarly, the production of high levels of IgE in response to parasitic infestations is a well recognised feature of the immune response.

Employing protein chemistry, structural studies and recombinant DNA technology, the possibility that a variety of antigens share common epitopes that give preferentially rise to an IgE response has been investigated, but despite intensive efforts and considerably increased information regarding the molecular structure of many common allergens no unifying feature has been proposed.

Our own studies, however, suggest an alternative explanation for the selective isotype induction observed for the above described antigens. Recently, observations from my laboratory have led to the development of a hypothesis that may help to explain the preferential selection of an IgE isotype commonly observed following exposure to certain antigens.

MANY ANTIGENS WHICH GIVE RISE TO AN IGE RESPONSE WILL TRIGGER MAST CELL SECRETION IN THE ABSENCE OF IGE SENSITIZATION

These findings became apparent when we tested a secretor variant of the rat basophilic leukaemia (RBL-3H3) cell-line (an accepted model system for the study of mucosal mast cell function), which had been transfected with the α-chain chain of the human high-affinity receptor complex [18]. Since human IgE only recognises primate receptors and no permanent cell-line is available that binds human IgE with high-affinity and responds to an immunological stimulus with mediator secretion, we chose this cell-line as host for α-chain gene transfection on the basis of earlier observations which had shown that γ-chains of rodent origin can facilitate the expression of human a chains in e.g. COS7 cells [19]. The high sequence homology between rat and human α-chain in the transmembrane domain suggested that the transfected human α-chain should form a functional complex with the subunits of the rodent receptor and that sensitization with hIgE should activate the host cell's signal transducing machinery following an antigenic stimulus. Experimental details regarding the engineering and biochemical characterisation of the transfected cell-line are shown in Figure 2.

When the transfected cells were sensitized with the serum from a bee venom phospholipase A2 sensitive individual (EC), mediator release could be demonstrated following challenge with purified bee venom phospholipase A2 (mellitin free). A typical bell-shaped dose response pattern was observed when the sensitized cells were challenged with increasing doses of antigen. Control experiments, where non-sensitized cells had been incubated with the same concentration of antigen in the absence of sensitizing serum also showed degranulation of mediators, but this time mediator release increased in response to antigen concentration (see Figure 3).

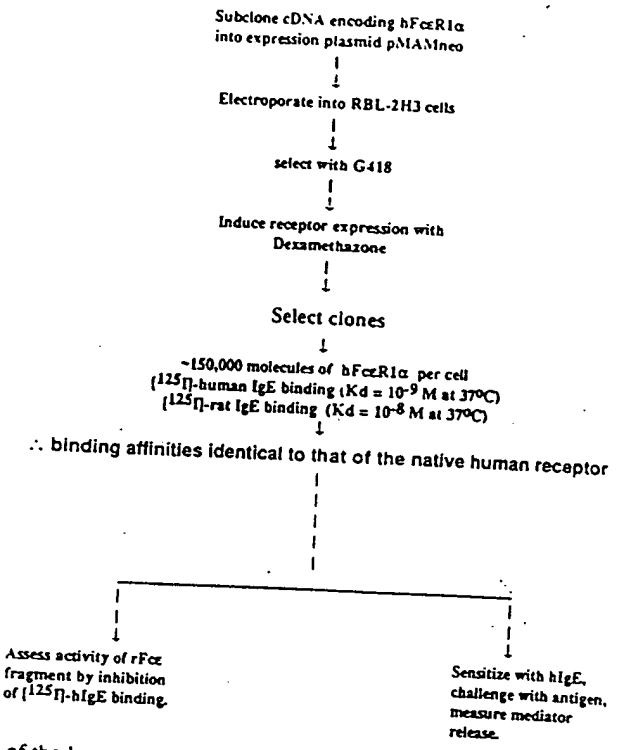

Figure 2. Expression of the human α-chain of the high-affinity receptor complex for immunoglobulin E in rat basophilic leukaemia cells.

The engineered cell-line binds human IgE with high-affinity. It was employed to map the FcεR1 binding site in hIgE, using a family of overlapping IgE derived peptides shown in Figure 1. Transfected cells respond to a human IgE-mediated antigenic stimulus with mediator secretion as shown in Figure 3.

Employing this cell-line, we were able to demonstrate that even in the absence of IgE several well defined allergens, (which in susceptible individuals give rise to an IgE response following the initial encounter) such as bee and vespid proteins, phospholipases, proteases from house dust mites and fungal spores, lectins present in pollen and grain, latex-associated products and spermicides, or aspirin based drugs, can trigger the release of substantial levels of mediators of the allergic response from these cells (see Table 1).

It has been known for more than a decade, that degranulation of mast cells and basophils can be induced by a variety mechanisms that do not involve IgE, e.g. mast cell secretion has been observed in response to bee and vespid venoms, or to drugs like codeine, morphine, or anaphylotoxins, and physical stimuli like pressure, heat, cold and sunlight [20,21]. Cross-linking by lectins that interact with carbohydrate residues on IgE or the receptors, oxygen radicals and proteases will induce the release of mediators [20]. Pollen and grain dust contain lectins in addition to proteases, which are also found in fungal spores, secreted by parasites, and several well characterised proteolytic enzymes have been isolated and cloned from house dust mites [22].

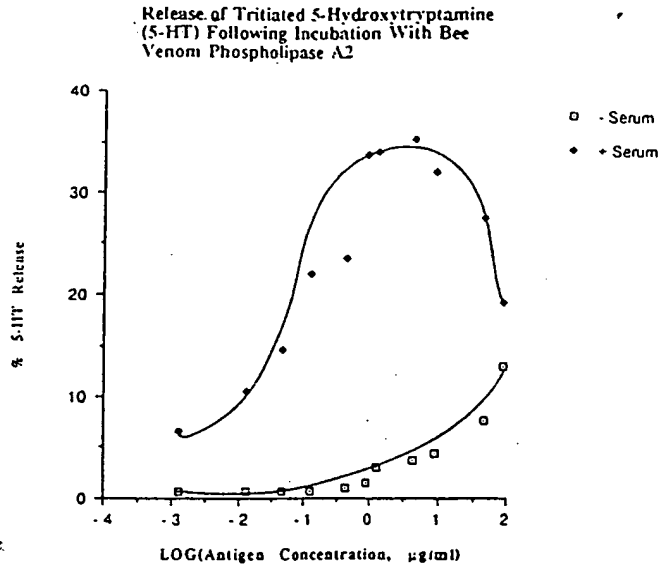

Figure 3. Transfected RBL-2H3 clones were incubated for 24h with or without serum (1:10 dilution) from a bee venom sensitive individual (EC) in the presence of 10^{-6}M dexamethazone and [^{3}H] 5-hydroxytryptamine. After washing, cells were challenged with antigen in the presence of 100mM 5' (N-ethylcarboxyamido)-adenosine (NECA).

[^{3}H] 5-Hydroxytryptamine was measured after a 15 min incubation period, release was corrected for background and expressed relative to the total [^{3}H] 5-hydroxytryptamine incorporated. For experimental details see ref.[18].

The molecular mechanism by which these agents trigger cell secretion is unknown. The pharmacologically active mediators that are released from the activated cell-line in response to these agents include histamine, serotonin, proteases, leukotrienes and cytokines.[17]. Of particular interest is the cytokine interleukin (IL)4, which is known to be essential for the induction of IgE by B-lymphocytes, and IL-6, which further enhances the IgE response induced by IL-4. Although IL-4 initiates the transcription of ε-chain germ line transcripts, a second B-cell activating signal, which may be involved in promoting recombinational accessibility is necessary in order to induce immunoglobulin gene class-switching [15]. *In vivo*, this signal is usually delivered to B-cells following interaction of the T-cell receptor/CD3 complex with MHC class II antigens. In the absence of such a stimulus, it can be replaced by a hormone/receptor interaction or a mitogenic stimulus [15].

Activated mast cells and basophils also secrete at least three proteases, the physiological function of which is unknown. These enzymes are serine endoproteases with a trypsin-like specificity. We have recently shown that after secretion following an immunological, non-immunological or oxide mediated stimulus, proteases released from the activated RBL cell-line can induce mediator secretion from cells of their kind (Helm *et al.*, paper in preparation). This secondary burst of the release of inflammatory mediators can be attenuated by the inclusion of protease inhibitor or substrates for serine

Table 1. Antigen-induced mast cell mediator release from RBL-2H3 cells in the absence of sensitization with antigen-specific IgE.

Antigen	Mediators Measured		
	5-HT	Protease	β-Hexosaminidase
Venoms, bee/wasp (1% suspensions)	++++	+++++	++++
Phospholipase A2 (bee venom), 1μg/ml	+	+	+
Phospholipase C (*B. cereus*) 1μg/ml	++++	++++	ND
House Dust Mite extracts, (freshly prepared) 1% suspensions	+++	+++	ND
House Dust Mite extracts, commercial sources, 1% suspensions	-	+/-	ND
Condom extract (1:400) (*Hevea brasiliensis*)	++	++	ND
Aspirin (25mg/ml)	+++	+++	ND
Influenza virus (5% suspension)	+	+	ND
Herpes simplex virus (5% suspension)	+	+	ND

Parent RBL-2H3 cells or clones transfected with the α-chain of the human high-affinity receptor complex were plated out in 24-well plates at 2 x 10^5 cells/well as described previously[18]. For the determination of antigen-induced mediator release, plates were incubated at 37°C for 15 min, cooled on ice, the supernatant was removed, spun at 200 x g (1 min) before liquid scintillation counting to measure [^{3}H]-5-hydroxytryptamine (5-HT) release. The ImmunoTech histamine enzyme immunoassay was used to quantify histamine release, and hydrolysis of toluene sulphonyl methyl ester was employed to monitor protease release [for exp. details see ref.[18]].

\+ - 3-8% mediator release
++ - 8-15% mediator release
+++ - 15-25% mediator release
++++ - 25-45% mediator release

proteases like the synthetic substrate p-toluenesulphonyl-L-arginine methyl ester (TAME) or the human IgE-derived pentapeptide (HEPP) [23] in the medium bathing the cells. This observation provides a self-evident explanation for the observed therapeutic effect of administration of HEPP to patients suffering from e.g. allergic rhinitis, initially attributed to be due to competitive inhibition of the IgE/FcεR1 interaction, but later held untenable [24].

It is well established that serine proteases mimic a number of insulin- and growth factor mediated processes including the initiation of cell division in quiescent cells [25,26]. Collectively this activity and the above mentioned observations suggest that IgE-independent stimuli can activate cells of mast cell/basophil lineage to deliver the IL-4 signal together with a mitogenic signal, thus providing a combination of stimuli which should be sufficient for the induction of IgE synthesis in B-cells. Under such conditions, any antigen present might be expected to give rise to the synthesis of an antibody of the IgE isotype.

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Protein and Cell Engineering of Components of the Human Immunoglobulin E Receptor/Effector System: Applications for Therapy and Diagnosis

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Abstract

Adaptive immune responses characterised by the synthesis of antibodies of the immunoglobulin E (IgE) isotype play an important role in type I hypersensitivity disorders and parasitic infestations, diseases which have an significant socio-economic impact wordwide. This paper considers potential applications of recent advances in our understanding of the origin of isotype specific immune responses which emerged as a result of cell and protein engineering studies on components of the human IgE/receptor/effector system. Furthermore, the identification of the receptor binding regions in IgE as a result of the development of a stable assay system has important applications for the design of rational therapeutic interventions in allergy and asthma, the treatment of mast cell tumours, and the establishment of procedures for the selective isolation of cells expressing the high-affinity receptor for IgE for functional studies.

Key words: immunoglobulin (Ig)E, high-affinity receptor for IgE, allergy, parasitic infestations

1. Introduction

An investigation of structure/function relationships in human (h) immunoglobulin (Ig)E and its receptors together with a study of the molecular mechanisms which cause IgE-mediated hypersensitivity reactions and stimulate immunity to parasitic infestations is very timely in view of the socio-economic impact of both diseases. In the developing world some 100 million people suffer from parasitic infestations, while in industrialised countries, the incidence of IgE-mediated allergies and asthma has more than doubled during the past 25 years [rev. in refs. 1-5].

At birth, IgE levels are either not measurable or exceedingly low. In normal adults, plasma levels of IgE rarely exceed 100 µg/l, but are elevated in allergic and parasitic disease. The sustained production of IgE antibodies in response to parasitic infestations is considered a beneficial immune defence mechanism, while the induction of IgE synthesis by a large number of seemingly diverse and inoccuous antigens is a pathological immune response which results in the development of type I hypersensitivity responses.

An outline of consequences of IgE-mediated target cell activation is shown in Fig. 1. Antibodies of the IgE isotype are usually synthesised and secreted from B lymphocytes in response to allergens or parasite proteins, although substances with suitable adjuvant activity can stimulate an IgE response to by-stander antigens [2,6]. Very little IgE is found in the circulation because IgE antibodies bind with high affinity to Fc receptors (FcεRI) found predominantly on mast cells and basophils, and with low-affinity to receptors (FcεRII) found on various inflammatory cells including macrophages and platelets. At any time, most IgE molecules are cell bound and extensively distributed on the surface of mast cells found in the mucosal lining of the eyes, lungs, skin and the intestine. These IgE sensitised cells are the major target organs in immediate hypersensitivity reactions. Following challenge with cognate antigens/allergens, they respond with the secretion of a wide spectrum of pro-inflammatory molecules, including histamine, prostaglandins, leukotrienes, proteases and chemokines. Cytokines released from IgE-activated mast cells and basophils include interleukin (IL)-4, which induces the expression of MHC class II and FcεRII molecules in target cells. These molecules also provide an up-regulatory feedback signal that stimulates IgE synthesis in B cells. In addition to causing the symptoms of the acute phase of the allergic response, they also induce, via the release of chemotactic mediators such as tumour necrosis factor (TNF) α, the recruitment of inflammatory cell sub-populations, which include eosinophils, macrophages and platelets, into the site of immediate hypersensitiviy, while IL-5 plays a key role in the activation of eosinophils. Eosinophil-mediated cytotoxicity depends on mast cell mediators and in the lungs of asthmatics and tissues invaded by parasites, eosinophils are found in close association with mast cells. Furthermore, oxygen metabolites, which are released from IgE-activated eosinophils, can induce mast cell secretion through an IgE-independent stimulus [1,7]. These mechanisms contribute to the clinical manifestations of the late phase of the allergic response and illustrate the importance of mediator release from mast cells and basophils in immediate and delayed hypersensitivity responses. An inhibition of the initial degranulation event should therefore be associated with wide ranging short and long term anti-inflammatory benefits [rev.in ref.1 & 2].

Despite intensive efforts, there are no effective medications to treat allergies and asthma. Currently available therapeutic interventions are inadequate and many are associated with undesirable and often severe side effects. Similarly, attempts to develop effective vaccination schedules for the treatment of parasitic infestations have had only limited success. An improved understanding of the interaction of the molecular

mechanisms involved in these disease processes should assist the development of rational therapeutic interventions.

2. Materials and Methods

2.1 Generation of rodent cell lines expression the ligand binding domain of the high-affinity receptor for human IgE

Rat basophilic leukaemia cell lines (RBL) expressing the human (h) α-chain of the FcεRI compex were engineered using as a host cell line a high secreting variant of the rat RBL 2H3 cell line [8], which expresses a functional receptor complex for rodent IgE. The h FcεRI α–chain gene was subcloned from pUC19 into the multiple cloning site of the vector pcDNA3 which supports constitutive expression of recombinant proteins in mammalian cells. Correct insertion was confirmed by gene sequencing. The plasmid containing the h FcεRI α-chain gene was transfected by electroporation into the RBL-2H3 cells [9] and is expressed as a functional unit with the rodent receptor on the cell surface. The generation and characterisation of the RBL 2/2/C cell line, which supports dexamethazone inducible expression of h FcεRI α, and the characterisation of IgE binding and secretory responses in native and transfected cells, has been described in earlier publications [8,9,11,12].

2.2 Identification of the high- and low-affinity receptor binding site in h IgE

The methodology has been described in earlier publications [1,10].

2.3 IgE-independent activation of mast cell mediators by potential allergens

The methodology has been described in earlier publications [11,12].

3. Results

3.1 Strategies for th d velopment of therap utic interventions in allergy and asthma

Although the ancient Egyptians already knew of sudden death as a result of bee stings, the dramatic rise in recent years in the incidence of allergic disease and IgE-mediated asthma in industrialised countries has stimulated the quest for the development of more effective rational therapeutic interventions in allergic disease [1-3].

One such approach focuses on the nature of the complementary binding site between IgE and its receptors. The binding of IgE to both types of receptors is a reversible process which sensitises, but does not induce mediator release until the ligand becomes aggregated, usually by cognate antigen, lectins or anti-IgE antibodies [1,12]. Soon after the discovery of the IgE antibody as the mediator of the allergic response, it was demonstrated that a h IgE fragment, which was prepared by papain cleavage and comprises amino acid (a.a.) residues 227-547 of the disulphide linked Cε2-4 dimer, can competitively inhibit the sensitisation of skin mast cells in passive cutaneous anaphylaxis (PCA) tests [13]. This initiated the search for progressively smaller Fcε peptides as potential blocking agents. In order to overcome the limitations imposed by proteolysis, IgE-derived peptides and chimaeric rodent/human IgE constructs were generated by chemical synthesis or recombinant DNA techniques with the aim of identifying the sequence requirements for the complementary interaction. There is still considerable discrepancy concerning the precise location of the FcεRI binding site in h IgE, although there is now a broad consensus that the binding region for both receptors is located in the Cε3 domain [rev. in ref.1]. This disagreement can be attributed largely to the fact that h IgE only binds to primate or human FcεRI, and in the absence of a permanent cell line, which expresses the h FcεRI complex, nearly all data reported before the 1990s, relied on the inhibition of the PCA reaction, which produced inconsistent results [rev. in ref.1].

In order to elimiate the problems associated with this temperamental assay system, we developed well-defined *in vitro* assays where the binding of IgE to the soluble extracellualr domain of h FcεRIα can be assessed [11,15]. In addition, we transfected the gene encoding the α–chain of the h FcεRI complex into RBL cells. A functional rodent FcεRI complex is expressed in these cells, which does not bind h IgE. It is made up of an a-subunit, which comprises the IgE binding site, a ß-subunit, and two disulphide linked γ-chains. We chose this cell line, which represents an accepted model system for the study of mucosal mast cell function, because earlier investigations had shown that γ-chains of rodent origin can facilitate cell surface expression of h α–chain gene products in e.g. COS7 cells [16]. In addition, the high sequence homology between rodent and human α–chains in the transmembrane domains suggested that the transfected h α–chain should form a functional complex with the ß and γ-subunits of the endogenous rodent receptor complex. Thus, sensitisation with h IgE should activate the host cell's signal transducing machinery in response to a h IgE-mediated antigenic stimulus. Figure 2 shows the technology employed in the engineering of RBL 2/2/C cells, which express ~100 000 h α–chains following receptor induction with dexamethazone and that of another variant, RBL J41, which supports constitutive expression of <10 000 h α–chains per cell. Transfected cells bind h IgE and support h IgE induced mediator release. The RBL 2/2/C cell line was employed to map the FcεRI binding site in h IgE, using a family of overlapping IgE-derived peptides, expressed in *E. coli*, shown in Figure 3. Furthermore, values for the kinetics of association and dissociation obtained from the *in vitro* assay

system (BiaCore) are in excellent agreement with studies where the binding to the receptor on transfected cells is assessed [14].

3.1.2 Identification of the FceRI binding site in h IgE: applications for the structure based design of anti-allergic drugs

Fig. 3 shows that the sequences common to all Fcε fragments capable of recognising FcεRI comprise Pro 343-Ser353 in the Cε3 domain. Further deletion from either the N- or C-terminal end is associated with a loss of receptor recognition [10]. The Pro343-Ser353 peptide blocks IgE/FcεRI binding with an IC_{50} in the mM range [1,17]. Such low affinity is commonly observed with linear peptides and attributed mainly to the ability of the peptide to adopt a large number of conformations in aqueous solutions. There is however evidence that appropriate conformationally restrained analogues can exhibit enhanced specificity and affinity. Viewed in the context of the model structure we developed for h IgE-Fc, the Pro343-Ser353 sequence has been computed to form an exposed loop [1,10,18], and this provided the basis of the disuphide bond constrained peptide shown in Fig. 4, which blocks IgE/FcεRI interaction in a competitive manner with an IC_{50} in the μmolar range. This increase in affinity suggests that this "lead" peptide may form the starting point for the development of low molecular weight anti-allergic drugs [rev. in ref.1].

Furthermore, when this peptide was employed to raise antibodies against the FcεRI binding region in h IgE, it was found that these antibodies bind to IgE in solution, but do not recognise receptor bound IgE and inhibit the binding of IgE to FcεRI [1,14]. The structural basis of this phenomenon, which appears paradoxical in view of the fact that IgE is a homodimer and antibodies are divalent, is unknown. It has been explained in terms of a bent conformation of IgE shown in Fig. 5, where the second ε-chain becomes inaccessible to an additional copy of the receptor, or to antibodies directed against epitopes in IgE that become masked following receptor engagement. As our study shows, such IgE epitopes may have applications as immunogens in the therapy of all IgE-mediated allergies through active immunisation irrespective of the nature of the allergen [rev. in ref.1].

The development of such vaccines is very timely in view of the dramatic rise in IgE-mediated allergies in recent years for which there exist no obvious underlying cause. Although genetic evidence indicates the importance of hereditary factors in immunity to parasites and susceptibility to develop IgE-mediated allergies, it appears probable that environmental factors play a decisive role in the current epidemic of allergic diseases since the gene pool of the population cannot have changed sufficiently to explain the recent increase in the incidence of type I hypersensitivity responses [2]. Although some observations point to a connection between the decline of infectious diseases and the rise in allergies and asthma [rev. in ref. 2,3], there is also compelling evidence that pollutants in air such as diesel exhaust particles, polyaromatic hydrocarbons, oxygen radicals produced by engine emissions or cigarette smoke, can have adjuvant activity and enhance IgE ongoing synthesis [19-25].

3.2 Uncov ring a link betw n the natur of substances that activat cells of mast cell/basophil lineag and the allergic respons

The exposure to several types of antigens, including pollen grains, mould spores, house dust mite and cockroach emanations, latex, fruit and nut associated substances or parasite secretions, gives rise with preference to the synthesis of antibodies of the IgE

isotype in susceptible individuals. However despite a large amount of information regarding the molecular structure of many allergens and parasite proteins, no unifying principle has been proposed that explains the nature of the isotype selection which consistently occurs in response to these diverse substances. Our own studies emerged with an unexpected alternative explanation for the selective isotype induction elicited by these antigens [12-26].

This became apparent when we employed the h α–chain transfected RBL cell line to study allergic sensitisation *in situ* as an alternative to the PCA test, since this procedure is associated with the inherent danger of boosting an already sensitised individual [27]. In order to assess the technology, we sensitised the cells with the serum of a bee venom sensitive individual (EMC) and, following challenge with the major bee venom phospholipase A_2 (PLA_2), we could, as expected, demonstrate mediator release. Surprisingly, however, control experiments, where non-sensitised cells had been incubated with the same concentration of antigen in the absence of the serum containing bee venom specific IgE, cells also responded with the degranulation of cellular mediators [11,12,26]. Further studies showed that only enzymatically active bee venom PLA_2, but not an inactive variant, is able to induce IgE-independent mediator release, including IL-4 from this cell line. Furthermore, only mice immunised with enzymatically active PLA_2, but not an inactive variant, produce high levels of PLA_2-specific IgE [11]. This suggested that the catalytic activity, manifested as IgE-independent mast cell secretagogue activity, determined the outcome of isotype specific immune responses.

This initial observation led to an extensive investigation into the IgE-independent activation of cells of mast cell/basophil lineage by potential allergens. Interestingly, potent hydrolytic enzymes, most of which are associated with catabolic pathways, have been isolated from nearly all sources of allergenic materials [28]. Similar proteins are secreted by parasites as part of the invasive process [11,12].

As summarised in Table 1, proteolytic and lipolytic enzymes from organisms as diverse as plants, fungi, house dust mites and schistosomes stimulate degranulation of cellular mediators from RBL cells and induce IL-4 synthesis and secretion. This IgE-independent cell activation is critically dependent on enzymatic activity since inactive forms of e.g. bee venom PLA_2 or the house dust mite protease *Der p I* do not induce mediator release [11,12]. Other classes of substances which induce mediators release and stimulate cytokine synthesis are lectins, including those present in natural latex and ragweed, virus associated proteins with protease or lectin-like haemagluttanin activity [12,26], or substances like the polycationic mast cell degranulating agents including mellitin, mastoporan, substance P, and compound 48/80. These are thought to activate heterotrimeric G proteins of the Rab family, which act as regulators of membrane fusion [29]. In addition, components in car engine emissions and cigarette smoke induce mast cell mediator release [30]. There is evidence that the former can act as adjuvants, since stimulation of ongoing IgE synthesis has been observed following exposure to diesel exhaust particles and polyaromatic hydrocarbons [21-23] and it is interesting to note that cigarette smoke contains many components also found in diesel engine emissions [22].

Although the RBL cell line presents a cellular model system for the study of mast cell function, we extended our investigation to assess the responses of preparations containing human lung (HLMC) and skin mast cells (HSMC) and basophils to confirm the commonality of our findings. Similar observations were made, indicating that we have identified an important biological principle underlying potential allergenicity [12].

4. Discussion and conclusion

Our results show that protein and cell engineering studies on components of the h IgE receptor/effector system have important applications for the diagnosis and therapy of allergic, parasitic and possibly also viral diseases in relation to the development of allergies [1,11,12].

4.1 Assessment of anti-allergic drugs

The development of stable cell lines, which express the ligand binding domain of h FcεRI and which respond to a h IgE-mediated antigenic stimulus with mediator release, led to the identification of the minimum sequence requirements for the binding to both receptors. This may form the basis of for the design of anti-allergic drugs, based on the structural motif contributed by the A-B loop in the Cε3 domain of h IgE. shown in Fig. 4. Both the *in vitro* assay system [14] and the FcεRIa transfected cell lines [9,27] can be employed for the screening and evaluation of potential blocking agents of IgE/receptor interaction and mast cell activation.

The engineering of a variant form of IgE ([IgE R16) [11, 15] which selctively recognises cells expressing the high-affinity receptor, but which does not bind to FcεRII/CD23 has potential therapeutic applications in the treatment of systemic mastocytomas when linked to an immunotoxin or radioactove isotope. In addition, it can be used for the selective isolation of cells expressing FcεRI for functional studies.

4.2 Design of vaccination schedules in allergic and parasitic disease

The demonstration that enzymatically active allergens and parasite proteins activate cells of the immune system to induce cytokine secretion has potential applications for the design of immunisation schedules in allergic and parasitic disease. Immunisation with active parasite protein and/or the use of IL-4 as an adjuvant may induce a protective immune response. Conversely, immunisation schedules employing biologically inactive allergens, anti-IL-4 antibodies, or adjuvants known to induce alternative Ig isotypes may prove effective for the treatment of allergies.

4.3 Development of new diagnostic tests to monitor allergic sensitisation

An *in situ* cellular assay system for the testing of allergic sensitisation offers an attractive and safe alternative to the skin prick test, which may carry the risk of boosting an already sensitised individual [16]. In addition, a considerable number of PCA tests are positive, although no allergen specific IgE can be demonstrated in the patient's serum [23]. This can problably be attributed to the IgE-independent secretagogue which we have shown to be associated with many sources of allergens and our assay system can clearly differentiate between these situations and eliminate potentially false positive results.

4.4 Development of an assay system to predict the potential allergenicity of environmental and occupational hazards

In addition, our observations indicate that the reason why diverse aero-allergens, environmental pollutants, parasite proteins or insect venoms induce and/or enhance IgE synthesis, is related to their ability to stimulate the release of cellular mediators, including IL-4. It indicates that any substance which induces the secretion of IL-4 must be considered as a potential allergen. The montoring of air quality involves measurements of NOx gases (nitrogen dioxide, nitric oxide, and sulphur dioxide), ozone, pollen counts and

particulate matter. No biological assay exists to assess the immediate and long term effects of these substances on cells of the respiratory tract. Risk assessment by means of a biological assay on cells which closely resemble the mucosal mast cells of the airways [2] is preferable to assessment of epidemiological evidence since this will facilitate pro-active rather than reactive responses when considering airborne allergens in the environment and at the workplace.

The demonstration of non-immunological mediator release from RBL and primary human mast cells and basophils indicate that common cellular responses to these substances occur in rodents and humans. This suggests that RBL cells can be employed to monitor potential allergenicity of occupational and environmental pollutants. The establishment of a cellular assay system to correlate air quality with potential allergenicity may have wide ranging applications in industry and the environment.

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Fig. 1. IgE-mediated cell activation and ist consequences in immediate and delayed hypersensitivity reactions. Following synthesis and secretion from B lymphocytes, IgE binds rapidly to high-affinity receptors. The initial interaction does not cause mediator secretion. This take place upon subsequent interaction of receptor bound IgE with cognate antigen. It initiates cell degranulation. Pharmacologically active mediators are rapidly released and these cause the clinical symptoms associated with type I hypersensitivity. In addition, chemokines secreted by IgE-activated mast cells and basophils activate and recruit inflammatory cells into tissues affected by immediate hypersensitivity responses. These cause the symptom associated with delayed hypersensitivity responses. Furthermore, via the secretion of IL-4 an up-regulatory feedback occurs on IgE synthesis by B cells. An inhibition of the initial sensitisation with IgE may therefore be associated with considerable ant-inflammatory benefits. The development of small molecules which block the initial docking of the ligand into the receptor is therefore an important goal of medicinal chemistry programs.

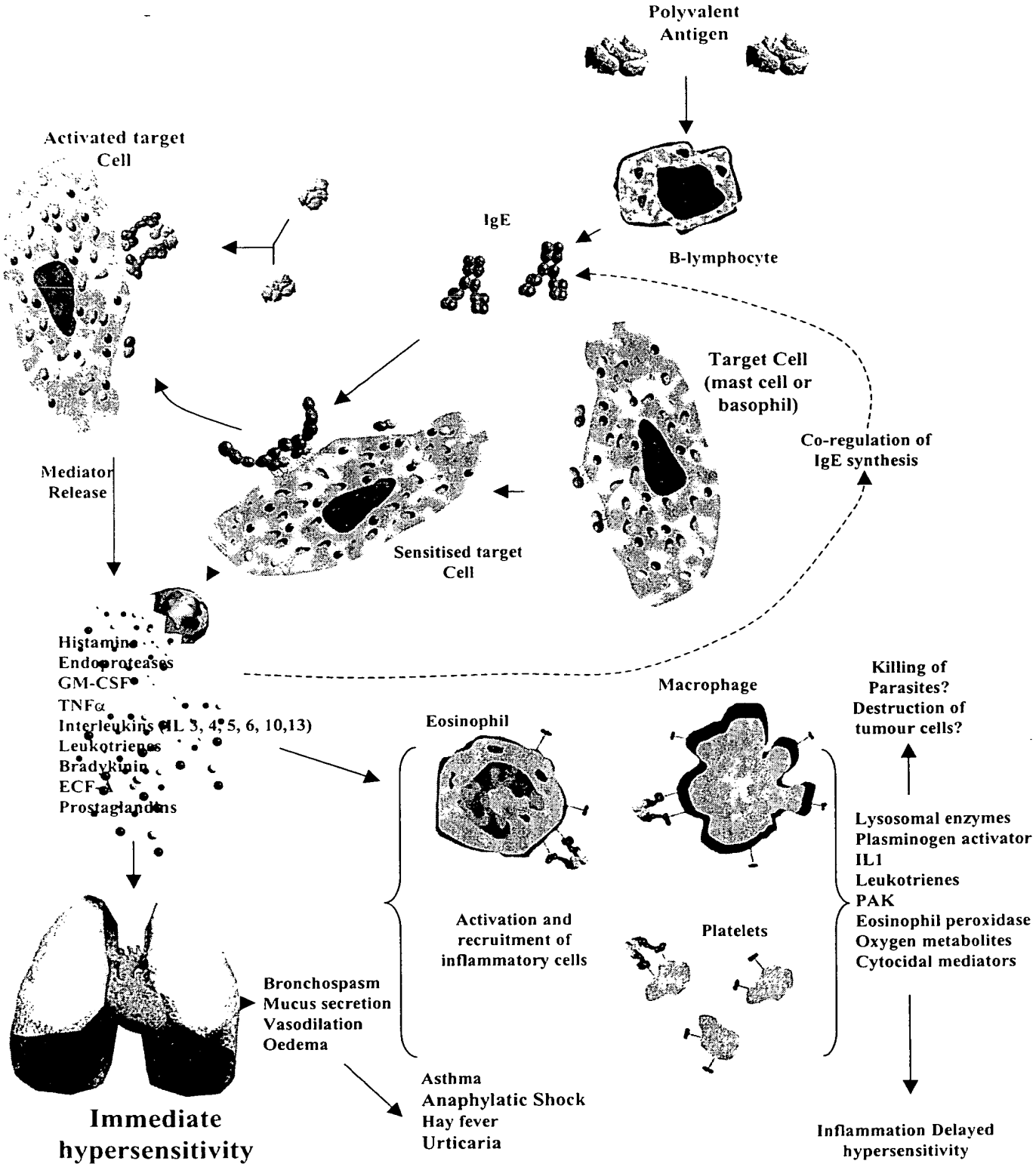

Polyvalent Antigen
Activated target Cell
IgE
B-lymphocyte
Target Cell (mast cell or basophil)
Co-regulation of IgE synthesis
Mediator Release
Sensitised target Cell
Histamine
Endoproteases
GM-CSF
TNFα
Interleukins (IL 3, 4, 5, 6, 10,13)
Leukotrienes
Bradykinin
ECF-A
Prostaglandins
Eosinophil
Macrophage
Killing of Parasites? Destruction of tumour cells?
Lysosomal enzymes
Plasminogen activator
IL1
Leukotrienes
PAK
Eosinophil peroxidase
Oxygen metabolites
Cytocidal mediators
Activation and recruitment of inflammatory cells
Platelets
Bronchospasm
Mucus secretion
Vasodilation
Oedema
Asthma
Anaphylatic Shock
Hay fever
Urticaria
Immediate hypersensitivity
Inflammation Delayed hypersensitivity

Fig. 2. Establishment of stable rodent mast cell line expressing the ligand binding domain of the human high-affinity receptor complex for IgE. The engineered cell lines support constitutive (RBL J41) and inducible expression (RBL 2/2C) of h FcεRIα. Cells were employed to map the FcεRI binding site in human IgE, using a family of overlapping IgE-derived peptides, shown in Fig. 3. Transfected cells respond to a human IgE-mediated antigenic stimulus with mediator secretion and have useful applications in diagnosis and for the screening of potential anti-allergic drugs.

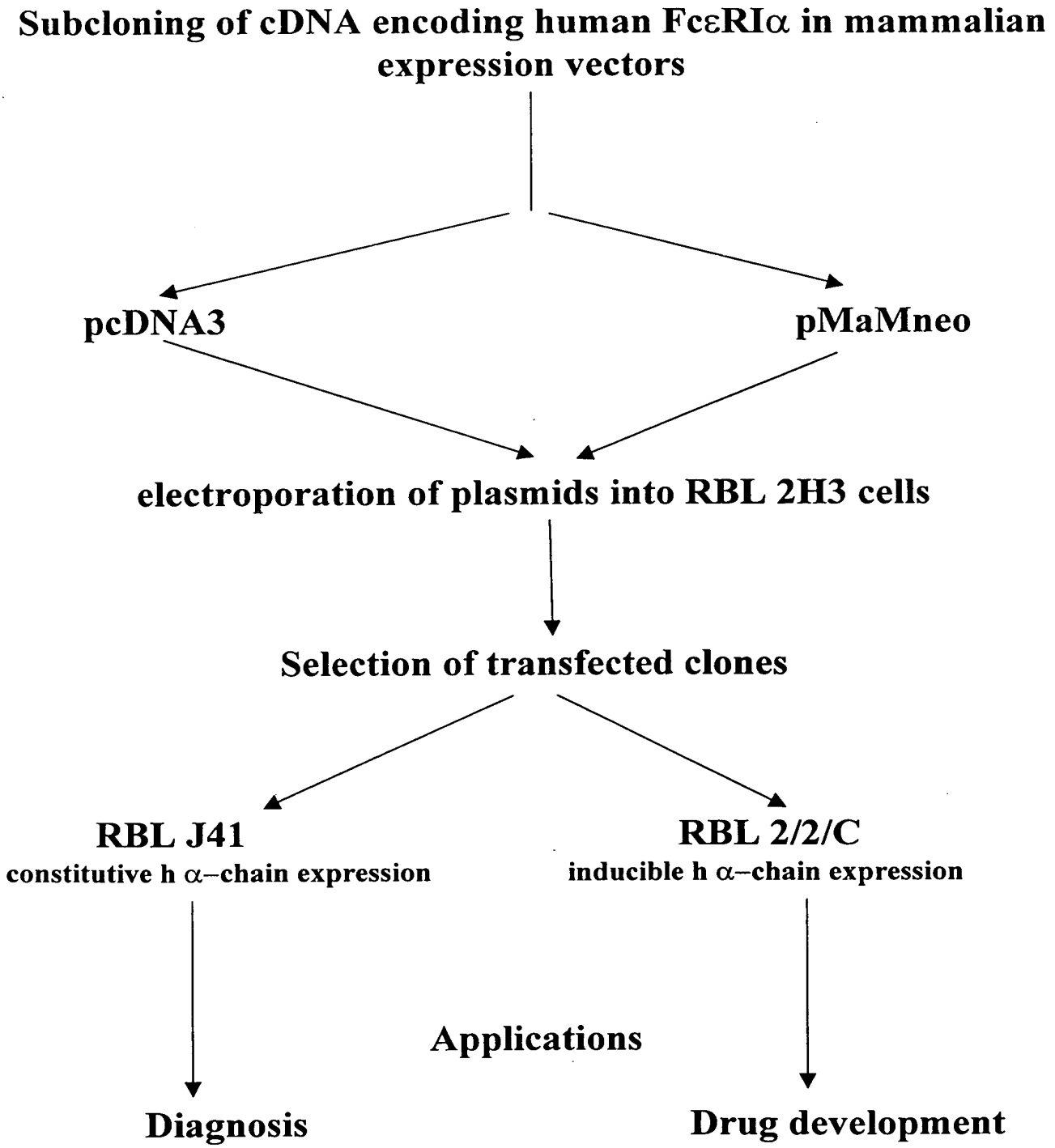

Subcloning of cDNA encoding human FcεRIα in mammalian expression vectors
pcDNA3
pMaMneo
electroporation of plasmids into RBL 2H3 cells
Selection of transfected clones
RBL J41
constitutive h α–chain expression
RBL 2/2/C
inducible h α–chain expression
Applications
Diagnosis
Drug development
Testing of allergen specific IgE from the serum of allergic individuals
Monitoring of potential allergenicity of environmental agents
Screening of potential anti-allergic drugs

Fig. 3. Identification of the receptor binding regions in human IgE. For experimental details see refs 1,7.

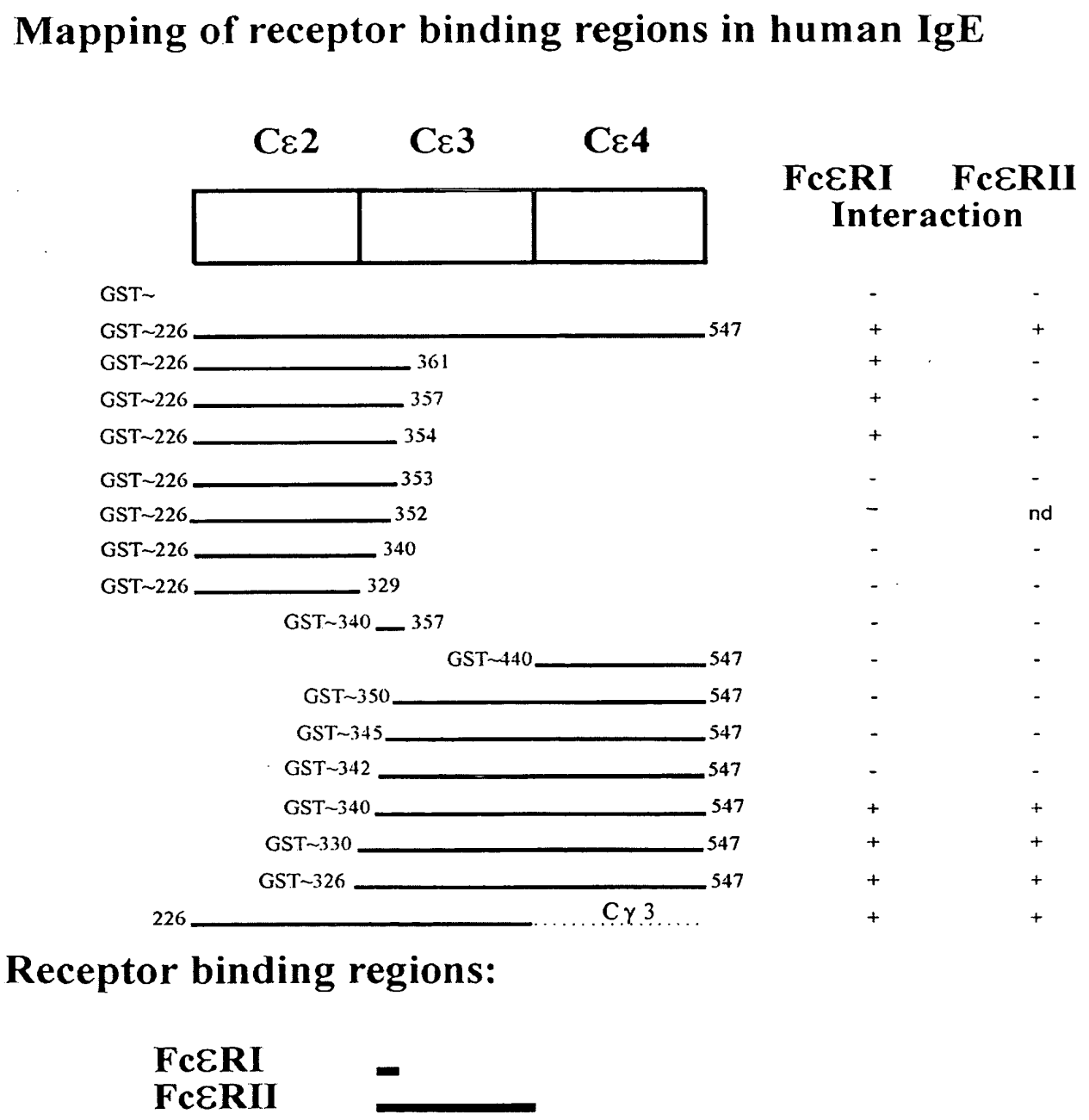


Fig. 4. Model structure of a cyclic peptide based on the A-B loop of h Cε3. The peptide blocks h IgE binding to to h FcεRIα with an affinity in the μmolar range and may form the basis for the development of blocking agents which inhibit the binding of IgE to FcεRIα.

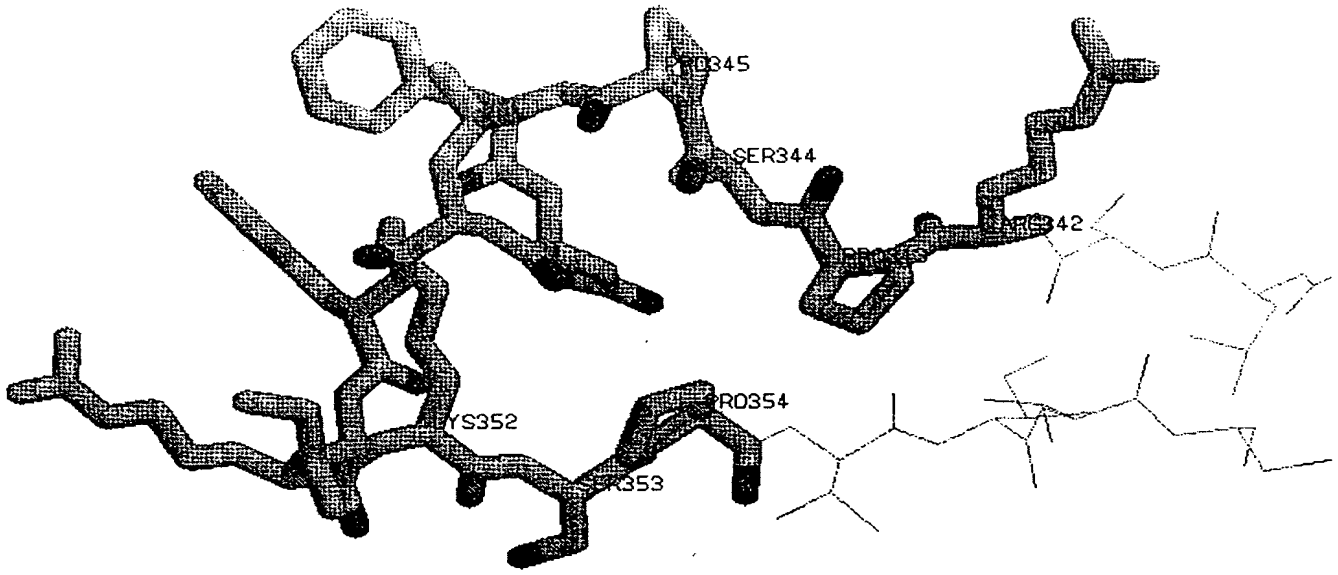


Fig.5. Structural models of IgE in coplanar (left) and bent conformation (right). A bent conformation of the IgE explains the observation that despite bilateral symmetry, the IgE molecule binds to FcεRIα and non-anaphylactogenic antibodies in a 1:1 stoichiometry. It indicates that the identification of epitopes recognised by the receptor of non-anaphylactic antibodies can lead to the design of peptide immunogens for active immunisation of asthmatics and patients at risk of anaphylactic shock.

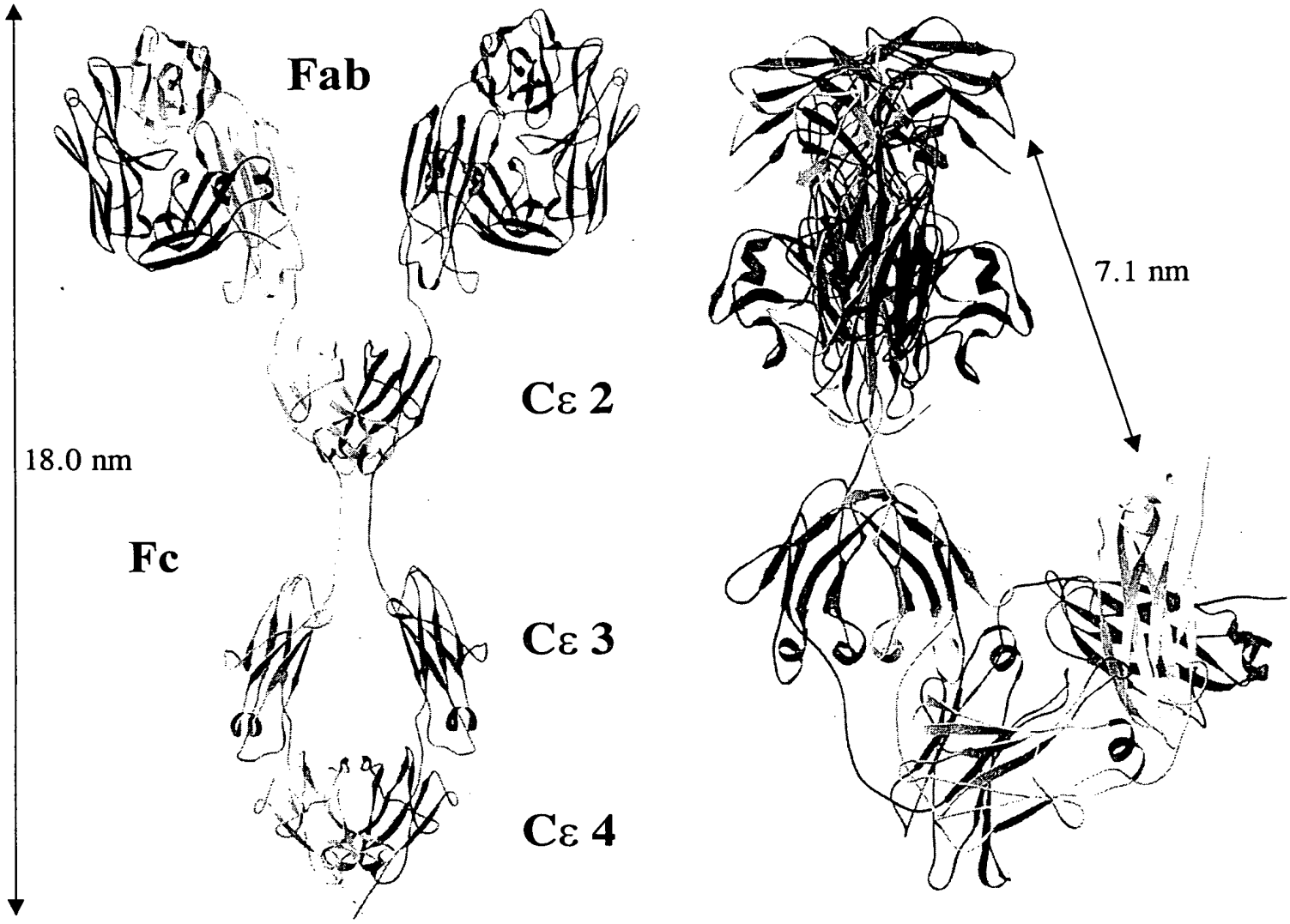


Table 1. Antigen-induced mast cell mediator release from RBL J41 cells in the absence of sensitisation with antigen specific IgE. Experimental details have been described in previous publications [11,12]. The enzymatic activity of all enzymes was tested before mediator release was assayed. In the absence of a quantitative assay for rat IL-4, the cytokine was detected by Western blotting.
d=detected, n.d. = not detected, n.t. = not tested. 5-HT = [^{3}H]-5 hydroxytryptamine.

	Mediators measured		
	5-HT	***Histamine***	***IL4***
Venoms, bee/wasp (1% suspensions)	**90**	**85**	**d**
Bee venom PLA2 (10µg/ml) recombinant enzymatically active	**17**	**17**	**d**
Bee venom PLA2 (10µg/ml) recombinant enzymatically inactive	**0**	**0**	**nd**
Der p I (3µg/ml) enzymatically active	**18**	**19**	**d**
Der p I (3µg/ml) enzymatically inactive	**0**	**0**	**nd**
Schistosomal protease (3µg/ml) enzymatically active	**17**	**19**	**d**
Apergillus protease (10µg/ml)	**21**	**24**	**d**
Natural latex (1:400) *hevea brasiliensis*	**15**	**20**	**d**
Condom extract (1:400) (Gossamer)	**12**	**18**	**d**
Hevein (1µg/ml) *hevea brasiliensis*	**8**	**9**	**nd**
Influenza virus F (5% suspensions)	**5**	**5**	**nt**
Respiratory syncytial vius (5% suspensions)	**7**	**5**	**nt**

new evidence about how allergies develop

allergies are on the increase: they affect about 30% of the world's population. Home-grown house dust mites have helped scientists at Sheffield University to understand more about how we become allergic to substances such as pollens, insect venoms and industrial pollutants.

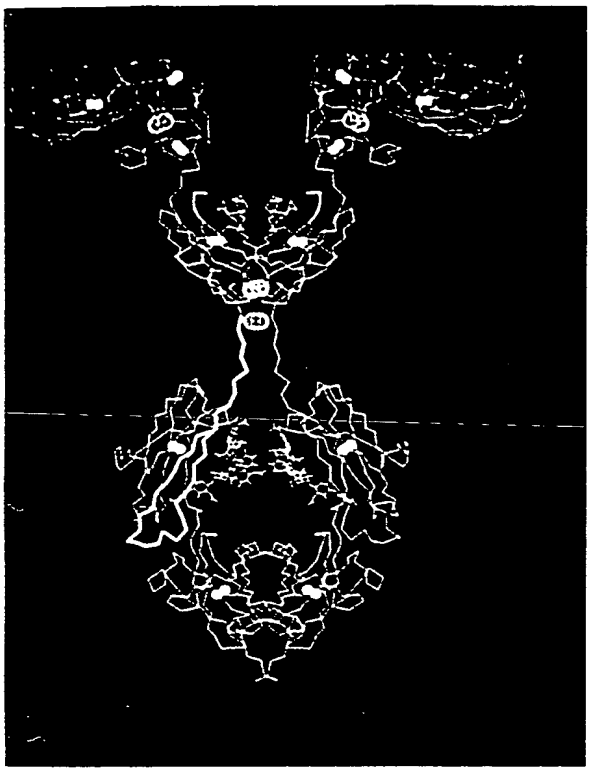
When Dr Birgit Helm and her colleagues set out with industrial partner, Euro/DPC Ltd, to develop a novel assay for measuring an important molecular interaction during the allergic response, they did not expect to emerge with a new theory of how allergies start. But that is what happened. Results from Dr Helm's laboratory show that compounds that cause allergies (allergens) interact directly with mast cells of the immune defence system in a hitherto unexpected way. The novel assay system has the potential for monitoring a patient's allergic sensitivities and may help in the design of novel therapeutic interventions in allergy. In addition it also presents the exciting possibility to predict the potential allergenicity of environmental agents by means of a cellular assay system.

Allergies are "unwanted" immune responses involving antibodies known as IgE. In simple terms, an allergy develops when an initial exposure to an allergen triggers production of IgE antibodies in the B lymphocytes of the immune system. These IgE molecules, each of which is specific for the allergen which triggered its production, migrate and bind to highly specific receptor molecules in the membranes of mast cells and other cells of the immune system – thereby sensitising these cells ready for any subsequent exposure to the allergen. These cells are distributed widely, for example, in the mucosal lining of the gut, lung and skin – major target organs in allergies. When subsequent exposure occurs, allergen molecules bind to the short "arms" of the Y-shaped IgE molecules which sit on their receptors with their long tail curved like a banana. Allergens effectively crosslink the receptors, and this in turn results in the secretion of mediators such as histamine, which cause the immediate clinical symptoms of allergy. Common allergic manifestations are hay fever, food allergies, or skin rashes, but in severe cases, asthma or anaphylactic shock ensues when breathing is obstructed and the blood pressure plummets and death can occur within minutes. In addition, allergens also stimulate the synthesis and secretion of mast cell cytokines, including interleukin 4, which is essential for IgE synthesis by B cells, thus amplifying an ongoing IgE response.

What led Dr Helm's group to identify another element in this process was the use of their new assay system* for monitoring the extent of cell sensitisation and levels of IgE in the blood. The assay uses cultured mast cells, originally derived from rat tissue. These have been genetically engineered to contain in their membranes the receptor molecule for human IgE antibodies. The blood from a colleague known to be allergic to bee stings was used to sensitise the assay cells; these were then "challenged" by the presentation of bee venom extracts. Predictably, the cells responded by secreting the signalling compounds that elicit the allergic response. But, quite unpredictably, control cells that were unsensitised also secreted these compounds when exposed to the allergen.

Birgit Helm decided to test whether this was a general property of allergens. It seemed to be so: many structurally different allergens triggered the unsensitised cells. But commercial preparations of the protease enzymes found in the droppings of house dust mites – very important allergens – failed to elicit this response. Undeterred, Helm

even the coffee mugs at Sheffield bear an IgE motif – Dr Birgit Helm with collaborator Dr Eduardo Padlan.



a stereodrawing of the structure of the human IgE molecule showing the short "arms" (top) which bind allergen and the region (white) which binds to the membrane receptor.

looked at the protease activity in these preparations and showed it was degraded; her research student Denise Machado then grew up her own colony of dust mites to obtain fresh supplies of "emanations". It worked, the purified mite proteases triggered unsensitised mast cells.

How this triggering is achieved is unclear. But the results show that a range of allergens, many of them proteins or even enzymes, initiate mast cell sensitisation directly by interacting with the cells and causing them to secrete signalling molecules that stimulate IgE production by the lymphocytes.

Unlike other responses of the immune system, production of IgE antibodies seems to offer no benefits – only the painful misery of an allergic reaction – so why do we have IgE antibodies? It seems likely that they offered an evolutionary advantage to our primitive ancestors. IgE antibodies protect against worms and other parasites, probably by reacting against parasitic proteases – the same types of enzymes that are the allergens in, for example, house dust mite or cockroach faeces. Modern living may have turned the advantage of making IgE antibodies to a disadvantage – and certainly the incidence of allergies is increasing. Dr Helm believes that environmental factors may be important in this. She has found that reactive oxides such as are found in car exhaust fumes, can trigger mast cells in a way analogous to traditional allergens. The new assay will allow closer examination of precisely who is allergic to what and why.

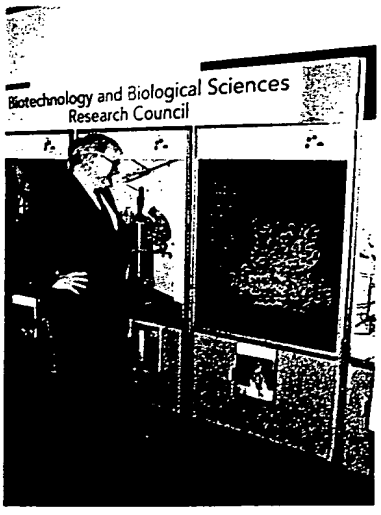
*Details of the science that led to this new assay may be found in the first issue of the newsletter of the BBSRC's Chemical and Pharmaceutical Directorate.

contact: Dr Birgit Helm
Krebs Institute for Biomolecular Research
Dept of Molecular Biology and Biotechnology
University of Sheffield
tel: 0742 768555

chemistry research for Britain

The BBSRC was one of the organisations supporting the "National Highlights of Chemical Science and Technology by Young Scientists from Academia, Government and Industry" in November. Keynote speaker was Sir John Cadogan FRS and the day comprised poster displays and short oral presentations. Several groups funded by the BBSRC presented examples of their work. The meeting was convened by Dr Eric Wharton.

the posters were judged on scientific content and presentation. Here, one of the judges, Dr John Bartlett (left) of BP Chemicals Ltd, discusses their poster with Dr Alison Watson of the Institute of Grassland and Environmental Research and Dr Neil of the Dyson Perrins Laboratory at the University of Oxford.



Sir John Cadogan FRS, Director General of the Research Councils looking at part of the BBSRC display.

Curriculum vitae: Birgit Anna Helm, MBB, University of Sheffield

Research

Since my appointment to a lectureship in Sheffield in 1989, I have built up an active research team investigating structure/function relationships in immunoglobulin (Ig)E and its receptors, and the molecular mechanisms underlying class specific immune responses, with particular reference to allergic and parasitic diseases. To support this research program I have:

- raised over £ 1.4 million (£ 1.2 million personally) from Industry (Euro/DPC, GLAXO/Wellcome, Pfizer, Eclagen), the Government Research Councils (MRC, BBSRC/DTI), and Charities (National Asthma Campaign, Wellcome Trust) for intramural and collaborative research with colleagues in the Chemistry Department and the Medical School;
- obtained funds from the European Union to co-ordinate a European network for a "Study of Regulatory Circuits in Allergic Disease", and support from NATO and the British Council for collaborative research with leading laboratories in the USA, Germany, and Switzerland;
- maintained a continuous publication record resulting in some forty research papers in refereed journals where I am predominantly first or senior author. One publication was rated outstanding and reprinted in a key journal for allergy research. Five international patents have been generated, two further patent applications are pending. Genetically engineered cell lines have been licensed by the pharmaceutical industry.

Standing in the profession

- Recently, I presented papers and chaired plenary sessions at scientific meetings: Frontiers in Allergy (Mainz, Germany, Sept. 97) and European Conference on Engineering and Medicine, (Warsaw, Poland, May 97),
- I have lectured at industrial and academic institutions including: Pfizer, GLAXO/Wellcome, Allergy Therapeutics, Peptide Therapeutics, at the Universities of Hannover, Southampton, Liverpool, Nottingham and Manchester, Guy's Hospital Medical School and King's College, London.
- In recognition of my contribution to IgE related research, I was an invited speaker at the International Jubilee Symposium "30 years with IgE", Stockholm, Sep. 1998.
- I am frequently invited to write review articles, edit scientific reviews, referee grant applications on behalf of the BBSRC, the NAC, the Wellcome and the Nuffield Trust (10-12 applications p.a.), and review publications for international journals (Int. Arch. Allergy, Allergy, Immunol. Today and Cell Biology International, ~15 publications p.a.),
- Research originating from my laboratory has received extensive coverage in the national and international press (ChemiDirect, bbsrc business, Look North, BBC Radio 5, BBC Sheffield, the World Service and the Daily Telegraph).

Teaching

- At undergraduate level, I contribute to and examine five lecture modules. I supervise three first year laboratory sessions. At second year level I deliver nine lectures each in two modules ("Molecular Biology Techniques", "Biochemical Messengers") to 190 and 100 students, and six lectures to 70 third year undergraduates ("Molecular Immunology"). I provide formative assessment to all students taking my second and third year modules.
- I am personal tutor to four first and five second year students. In addition, I contribute twelve academic tutorials to first and second year students. In 1998, I supervised six (average 3) third year undergraduates for laboratory project work.
- I participated in a M.Ed. Course provided by the University to stimulate the development of innovative teaching practices and to address the challenge presented by the teaching of large groups. I have also developed a computer based teaching and learning package on Allergy to complement lectures in the third year Molecular Immunology Course.
- Since 1989, I have supervised 15 postgraduate research students. Ten students have been awarded (one submitted their theses for) the degree of Ph.D. All theses were submitted within the statutory period imposed by the Research Councils. Two of my research students received prizes for work carried out under my supervision (International Academy of Allergology & American Association of Clinical Immunologists).

Administration

- I am course co-ordinator of three third year modules. I allocate both laboratory and library research projects to 165 students for supervision by some fifty members of academic staff. The laboratory and library modules represent the core of final year teaching (40 credits);
- I am a member of four postgraduate committees monitoring progress of research students supervised by colleagues, and I act as examiner in connection with the award of the Ph.D. degree;
- I am a member of the departmental biochemistry and genetics teaching groups, and a group concerned with improvements in teaching quality;
- I am departmental representative for students spending a year abroad (e.g. Erasmus students).
- I have organised postgraduate courses with invited external lecturers supported by funds I raised from industry;

1. PERSONAL DETAILS

SURNAME:	HELM	**FORENAMES:**	Birgit Anna
DATE OF BIRTH:	04.01.1946	**DEPARTMENT**	Mol. Biol. & Biotech.

EDUCATION AFTER SCHOOL:

Norwood Technical College, London		1968-1970
City of London Polytechnic		1970-1972
Bedford College, Univ. of London	(undergraduate)	1972-1975
ibid	(postgraduate)	1976-1978

QUALIFICATIONS:

O.N.C.	1970
H.N.C.	1972
B.SC. (Biochemistry, Class 1)	1975
Ph.D. (Biochemistry)	1980

MEMBERSHIP OF LEARNED SOCIETIES:

Biochemical Society
British Society for Immunology
International Society for Molecular Recognition

CURRENT APPOINTMENTS:

Lecturer, Dept. Molecular Biology & Biotechnology — 1989
University of Sheffield, S10 2TN
Senior Lecturer, ibid — 1999

PREVIOUS APPOINTMENTS

Research Associate — 1983-1988
Department of Biophysics, King's College, London

Research Associate — 1982
Texas A & M University, College Station, Texas, USA

Lecturer (temporary) — 1978-1981
Bedford College, University of London

Medical Laboratory Technician — 1968-1972
University College, London

2. RESEARCH

- **(1) Structure/function relationship in human immunoglobulin E and its receptors**
- **(2) Design of rational therapeutic interventions in allergic disease**
- **(3) Development of biological assay systems to monitor potential allergenicity of aero-allergens and pollutants**

RESEARCH GRANTS AND CONTRACTS

Funding obtained during the 1998/9session:

- **Licensing fee (non-exclusive) for genetically engineered mast cell line**

Sponsor	Peptide Therapeutics
Dates:	6/99-5/2000
Value	£ 10,000

- **A dog model of Blocking IgE:FcεRI interaction**

Sponsor	Allergy Therapeutics
Dates:	1/10/99-30/9/2002
Value	£ 126 000

- **Structure/function relationship in human IgE and its receptors**

Dates	10/98-9/2001
Funding body	BBSRC/CASE with Biovation
Value	£51,000

- **Structure based design and synthesis of antagonists of the allergic response**

Dates:	10/98-8/2000
Funding body	Wellcome Trust
Principal investigators	Spivey, A.C. Dept. Chem., Sheffield & B.A. Helm, MBB
Grant value	£ 49,073, supplementary support to

- **Structure based design and synthesis of antagonists of the allergic response**

Dates:	11/97-10/2000
Funding body	Wellcome Trust
Principal investigators	Spivey, A.C. Dept. Chem., Sheffield & B.A. Helm, MBB
Grant value	£ 147,812

Research funds raised since my appointment to Sheffield between 1989 and 1997

- **Protein Engineering of the high-affinity receptor complex for immunoglobulin E**

Dates:	10/97-9/2000
Funding body	National Asthma Campaign
Principal Investigator	B.A. Helm
Grant value	£126,091

- **Structure-function studies on human IgE and its receptors (renewal)**

Dates	6/97-5/99
Funding body	NATO
Principal investigators	B.A. Helm & E.A. Padlan (NIH, Bethesda, USA)
Grant value	£ 4000

- **Large scale expression of human IgE-Fc and receptor fragments for structure determination**

Dates:	5/97-4/98
Funding body	MRC
Principal Investigator	B.A. Helm

Grant value £ 39,184

- **An investigation into regulatory circuits in allergic disease**

Dates	10/94-3/98
Funding body	European Community
Principal Investigator	B.A. Helm
Grant value	ECU 150,000

- **Molecular basis of the allergenicity of bee venom phospholipase A_2**

Dates	3/96-2/97
Funding body	British Council
Principal Investigators	B.A. Helm & M.Suter (Zurich)
Grant value	£ 2000

- **Development of an assay system for the screening of potential allergenicity**

Dates:	10/95-9/98
Funding body	Health & Safety Executive
Principal investigator	B.A. Helm
Grant value	£ 21,600

- **Structure-function studies on human IgE and its receptors**

Dates	6/95-5/97
Funding body	NATO
Principal investigators	B.A. Helm & E.A. Padlan (NIH)
Grant value	£ 4000

- **Signal transduction in cells of mast cell lineage**

Dates	10/94-9/97
Funding body	GLAXO
Principal Investigator	B.A. Helm
Grant value	£ 49,500

- **Expression of the ligand binding domain of human FcεRI in *Pichia pastoris***

Dates	10/93-9/96
Funding body	Pfizer U.K.
Principal Investigator	B.A. Helm
Grant value	£ 25,000

- **A study of gene expression in releasing and non-releasing variants of the rat basophilic leukaemia cell line**

Dates	10/93-9/96
Funding body	National Asthma Campaign.
Principal Investigator	B.A. Helm
Grant value	£ 102,202

- **Expression of components of the IgE/receptor/effector system for structure/function determination**

Dates	1/91-12/94
Funding body	SERC/DTI/EURO DPC (LINK)
Principal Investigator	B.A. Helm
Grant value	£ 417,560

- **Recognition of class-specific Fc-receptors by human IgE**

Dates	7/89-6/92
Funding body	MRC/ EURO DPC
Principal Investigator	B.A. Helm
Grant value	£ 216,865

2.3 PUBLICATIONS

I am co-inventor of five patents and some 40 research papers, most of which were published in refereed international journals, including Nature, Proc. Natl. Acad. Sci., J. Immunol., J. Biol. Chem., Eur. J. Immunol., which have an impact factor of 6 or above.

3. TEACHING

3.1 CURRENT UNDERGRADUATE AND POSTGRADUATE TEACHING

Currently, I supervise 5 postgraduate research students (4 full-time and one part-time). I act as examiner in connection with the award of research theses and I lecture on a M.Sc. degree course in Molecular Immunology in collaboration with Sheffield Hallam University.

Undergraduate teaching involves lecturing (nine lectures and one tutorial) to 190 second year students on a course entitled "Molecular Biology Techniques", nine lectures and two tutorials to 80 second year students on a course entitled "Biochemical Messengers", six lectures to 65 third year students on a course in "Molecular Immunology". I also supervise three laboratory classes for 186 first year students and laboratory research projects for six third year undergraduate students. I am tutor to four first and five second year undergraduate students and hold academic tutorials (one per months) to second year undergraduate students.

3.2 PREVIOUS UNDERGRADUATE AND POSTGRADUATE TEACHING

There has been a continuos increase in my undergraduate and postgraduate teaching load during the past five years.

3.3 RESEARCH SUPERVISION

Since 1990, I have supervised fifteen post-graduate research students (thirteen full-time and two part-time). There is a monthly one hour journal club for postgraduate research students and weekly laboratory meetings. Ten students have been awarded the degree of Ph.D (one further thesis has been submitted). All full-time home students I supervised completed their theses in less than four years and no re-submissions were required. Two of my research students received prizes for work carried out under my supervision.

3.4 TEACHING INNOVATION AND DEVELOPMENT

In collaboration with my former research associate, N. Rhodes, we developed a computer based teaching and learning package on allergy to complement lectures in the third year undergraduate Molecular Immunology Course. This session I enrolled in a M.Ed. Course offered by the University. My aim is to study and implement innovative teaching practices and to address the challenge presented by teaching large groups.

4. ADMINISTRATION

As course co-ordinator for three third year modules, I am responsible for the co-ordination of the Molecular Immunology module, and the allocation of both laboratory and library research projects to 165 undergraduate students for supervision by some fifty members of staff. With 50 credits, these modules represent the core of final year teaching. I am a member of the departmental biochemistry and genetics teaching group, and a group concerned with improvements in teaching quality. In addition, I am a member of four post-graduate committees monitoring the progress of research students, act as examiner in connection with the award of Ph.D. theses. I have organised postgraduate courses with invited external lectures, supported by funds I raised from industrial sponsors. I am Departmental representative for contact with 'Year Abroad' students (1993 – present).

5. PROFESSIONAL ACTIVITIES

I have presented papers and chaired plenary sessions at several national and international meetings in Britain, Germany, Switzerland, USA, Canada and Japan (Germany, Poland and Sweden during the 1997/8 session). I also presented papers at major industrial and academic institutions in this country and overseas. In recent years, research originating from my laboratory has received considerable coverage in the national and international press.
I referee grant applications on behalf of the MRC, BBSRC, the Wellcome Trust and the National Asthma Campaign. I review manuscripts for Immunology Today, International Archives of Allergy and the European Journal of Allergy and Clinical Immunology and I am a co-editor of "Current Opinion in Biotechnology", and editor in chief elect of

Reviews in Protein Engineering/Wensum Academic Press. I have accepted an exclusive Consultancy with Peptide Therapeutics (15 days p.a.) to advise the company on allergy related research.

5.2. PUBLICATIONS

6.1 Book Chapters

J. 1. Development of Structural Models For a Study of the Interaction of Human IgE with Class-specific Fc-receptors.
Eduardo A. Padlan and Birgit A. Helm. In Allergic Mechanisms and Immunotherapeutic Strategies pp 1-15. Ed. A.M. Roberts and M.R. Walker. 1997 John Wiley and Sons Ltd.

P 2. Protein Engineering of IgE antibodies: Potential applications.
Helm, B.A., Ling, Y., Mackie, S., and Padlan, E.A. In Molecular Diagnostics: Research towards applications pp 312-332. Ed. A.M. Roberts and M.R. Walker. 1993 Blackwell Scientific Publications.

J 3. IgE in allergic inflammation
Marsh, P., Helm, B.A., and Gould, H.J. In Pharmacia Allergy Research Foundation, pp 26-31. Ed. S.G.O. Johannson 1988.

J. 4. Transfer of maternal proteins to a developing embryo
Cheesman, D.F. and Helm, B.A. pp 637-642. Eds. Vassileva Popova, J.G. and Jensen, E.V. 1979. Plenum Press N.Y.

In Press

5. Regulation of IgE synthesis
Helm, B.A. In Atlas of Asthma. Eds. Holgate S. and R. Djukanovic, 1999 Parthenon Publishing, in press.

6.2 Publications in refereed journals and conference proceedings

P = principal author J = joint author I.F. = impact factor of journal N/A not available

1. Sayers, I. and B.A. Helm (1999) Structural basis of human IgE-Fc receptor interactions. *Clin Exp Allergy 29: 585-94* (IgE model structure on front cover of journal); (P/ I.F.3.8).

2 I. Sayers, S.A. Cain, J.R.M. Swan, M.A. Pickett, P.J. Watt, S.A. Holgate, E.A. Padlan, P. Schuck and B.A. Helm (1998) Amino acid residues that influence FcεRI mediated effector functions of human immunoglobulin E. *Biochemistry* **37**, 16152-16164, (P/ I.F.4.8).

3 B.A. Helm, I. Sayers, S.A. Cain, J.R.M. Swan, L.J. Smyth, M. Suter, D.C. Machado. A.C. Spivey, E.A. Padlan (1998) Protein and cell engineering of components of the human immunoglobulin E receptor/effector system: applications for therapy and diagnosis. *Technology and Health Care* **6**, 195-207, (P/ I.F. 1.6).

4 B.A. Helm, I. Sayers, E.A. Padlan, J.E. McKendrick, and A.C. Spivey (1998) Structure/function studies on IgE as a basis for the development of rational IgE antagonists. *Allergy* **53**, 85-90, (P/ I.F.2.0).

5 Helm, B.A., Spivey, A.C. and Padlan, E.A. (1997) Peptide blocking of IgE/receptor interaction: Possibilities and pitfalls. *Allergy* **52**:6-13, (P/ I.F.2.0).

6 Machado, D.C., Horton, D., Harrop, R., Peachell, P.T. and Helm, B.A. (1996) Potential Allergens stimulate mediator release and interleukin-4 synthesis from cells of mast cell lineage in the absence of sensitisation with antigen-specific IgE. *Eur. J. Immunol.* **26**:2972-2980, (P/ I.F.6.0).

7 Helm, B.A., Sayers, I., Higginbottam, A., Machado, D.C., Ling, Y., Ahmad, K., Padlan, E.A. and Wilson, A.P.M. (1996) Identification of the mast cell binding region in human IgE. *J. Biol. Chem.* **271**:7494-7500, (P/ I.F.7.4).

8 Dudler, T., Machado, D.C., Kolbe, L., Annand, R, Rhodes, N., Gelb, M., Koelsch, E., Suter, M. and Helm, B.A. (1995) A link between catalytic activity, IgE-independent mast cell activation and allergenicity of bee venom phospholipase A 2. *J. Immunol.* **155**: 2505-2513. (P/I.F. 7.6).

9 Bingham, B.R., Monk, P.N. and Helm, B.A.(1994) Defective protein phosphorylation and Ca++ mobilisation in a low secreting variant of the rat basophilic leukaemia cell line. *J. Biol. Chem.* **269**:19300-19306, (P/7.4)

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13.2 Other Publications

Co-editor with E.A. Padlan of *Current Opinion in Biotechnology* **8** No 4, 1997.

P Editorial overview: Birgit A. Helm and Eduardo A. Padlan. Protein Engineering: From plants to animals, from big to small, from outside to inside and other advances. *Current Opinion in Biotechnology* 1997, **8:**397-399.

13.3 Research in progress and future perspectives

Allergic diseases, characterised by an increase in levels of antibodies of the IgE isotype, affect some 25% of the population in industrialised countries, and more than 10% of children suffer from IgE-mediated asthma. Currently, there are no satisfactory therapeutic interventions and the disorder has a profound socio-economic impact. The cost of disease management in Britain is in excess of £ 1 billion p. a. There is ample evidence that the molecular mechanisms which stimulate immune responses to parasitic infestations (another economically important disease) are also linked to the induction of IgE responses. The overall aim of my research programme is to generate information to assist the development of rational therapeutic interventions in allergic and parasitic disease.

Research is currently in progress

- to identify the complementary site of interaction between IgE and its high and low affinity receptors. As a result of our identification of the high-affinity receptor binding region in IgE, we have designed a "lead compound" (patent filed with E.A. Padlan, NIH, Bethesda, USA) which blocks IgE/receptor interaction. The further development of this lead compound (in collaboration with Dr. A.C. Spivey, Dept. Chemistry) will benefit from structure/function studies on the extracellular domains of the high- and low-affinity receptors for IgE, which we have over-expressed (in collaboration with Dr. M. Attwood, MBB). Structure studies by nmr (in collaboration with Dr. J. Waltho, MBB) and crystallisation trials have been initiated;

- to develop an allergic human SCID mouse model system (in collaboration with Dr. K. Else, Univ. of Manchester) for the *in vivo* assessment of anti-allergic and anti–parasitic drugs; which we are developing in collaboration with Dr. A.C. Spivey, (Dept. Chemistry, Sheffield) and Prof. A. Wilson, Univ. York);

- to establish a biological assay system to assess the potential allergenicity of allergens and environmental pollutants with collaborators at the Fraunhofer Institute in Hannover, Germany (Drs. M. Aufderheide and Dr. A. Emmendoerfer) and colleagues at ScHARR and the Health and Safety Laboratory, Sheffield (Prof. T. Higenbottam and A. Curran). Such an assay system has important applications in clinical and occupational medicine.

OTHER INNOVATIVE AND CREATIVE ACTIVITIES

7.1. PATENTS

1. Polypeptide competitor for human IgE (with Prof. H.J. Gould.)
   International patent 899604292.

2. Low-affinity IgE competitor (with Prof. H.J. Gould & Dr. P.B. Marsh)
   International patent 87904292.

3. Improvements relating to allergen testing and diagnosis (with Drs. P.Wilson, D. C. Machado, C.E. Pullar, and A. Camp)
   International patent GB/93/02430.

4. Inhibition of IgE-mediated allergies by a human IgE-derived oligopeptide (with Dr. E.A. Padlan)
   USA Provisional Patent Application 29768, filed Dec. 1996.

5. Applications of IgE variant Gly352
   Provisional Patent Application, filed Oct. 1998

6. Applications of IgE variant Gly333
   Provisional Patent Application, filed Oct. 1998

Assessment of the molecular basis of pro-allergenic effects of cigarette smoke.

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Abstract

Epidemiological studies point to a link between smoking and increased risk of immunoglobulin E-mediated allergies and asthma. The molecular basis underlying cigarette smoke related respiratory disorders are ill defined, but it is known that mast cells in the mucosal lining of the airways are an important reservoir of pro-inflammatory mediators, which play a pivotal role in the development of these diseases. The development of a novel cell exposure unit facilitated a study of mast cell responses to pollutants in cigarette smoke at the cell/air interface. Our study shows that cigarette smoke, but not filtered clean air, induces the release of mediators of type I hypersensitivity responses and stimulates the synthesis of pro-inflammatory cytokines, including interleukin (IL)-4, 5, 10, and 13 and tumor necrosis factor (TNF)α in cells of mast cell lineage. This explains how exposure to pollutants present in cigarette smoke can induce the pathophysiological responses associated with allergy, IgE-mediated and IgE-independent asthma since IL-4 and IL-13 induce class switching to IgE, and IL-13 has recently been identified as the key mediator of IgE-independent asthma.

Key words: cigarette smoke, mast cell degranulation, pro-inflammatory cytokine synthesis.

Introduction

Increased cancer risk as a result of long term exposure to cigarette smoke is well established. In addition, it has been extensively documented that cigarette smoking is responsible for initiating and/or exacerbating a variety of respiratory disorders, including chronic obstructive pulmonary disease, emphysema and asthma (1-5). Active and passive smoking also increases the risk of development of occupational allergies, and parental smoking is linked to increased airway hypersensitivity and sensitization to common aero-allergens in children (5-8). The molecular mechanisms inducing cigarette smoke related disease states are not understood. However the role of both interleukin (IL)-4 and IL-13 in the induction of immunoglobulin (Ig)E responses is well established and recently, evidence has been obtained which points to a key role of IL-13 in causing the symptoms of allergic asthma, which is independent of IgE and IL-4 (9,10).

Because mast cells are extensively distributed in the mucosal lining of airways and an important source of potent mediators of airway inflammation (11), we have monitored histamine secretion and cytokine synthesis in response to cigarette smoke. Preliminary investigations demonstrated histamine release from human lung mast cells (HLMC) and rat basophilic leukemia cells (RBL) (which present an established model system for the study of mucosal mast cell function) exposed to cigarette smoke containing air in a public bar. No degranulation was observed from cells maintained in a smoke free room, under otherwise identical conditions. The similarity of responses of HLMC and the RBL cell line to pollutants in cigarette smoke (and other environmental allergens (12,13), justify the use of the RBL J41 cell line as a model system of

mast cell responses to investigate the effect of cigarette smoke under more detailed and rigorously controlled exposure conditions and obviates the need to prepare primary HLMC [12,13]. Most importantly, our assay system facilitates direct exposure of cells maintained at a liquid/air interface to freshly generated cigarette smoke in the absence of an intervening layer of tissue culture medium (14). The experimental conditions, therefore, closely resemble cell exposure to the environment at mucosal surfaces.

Experimental Section

Exposure system. The design of the exposure chamber and dilution systems, including smoke generation and composition, have been described in detail elsewhere (14). Cells are cultured on a collagen substratum at a liquid/air interface and subjected over a period of up to 15 min (equivalent to the duration of inhaling one cigarette) to periodic exposure to a smoke containing air phase. The tissue culture unit allows variation in the exposure protocol with regard to smoke concentration and duration of exposure.

Cell maintenance and monitoring of cellular responses. The preparation of dissociated cell suspensions containing HLMC and the culture conditions for the RBL cell line have been published (12). Release of histamine, 5-hydroxytryptamine (5-HT), or β–hexoseaminidase (β–hex), as an index of degranulation, and lactate dehydrogenase (LDH), as an indicator of cytotoxicity, were determined using standard assays (12). RBL-J41 cells were plated onto collagen inserts of six well plate 'transwells' (Costar) at 2.5 x 10^6 cells/well, 18-24 hrs before transfer of collagen transwells with adhering cells into the smoke exposure chamber. Exposure conditions were optimized to obtain high levels of mediator secretion, while minimizing cytotoxic effects. The smoke dilution setting of the apparatus, which is variable between 1:8 and 1:200, was set at 1:100 dilution with a cigarette smoke rate of 1 inhalation per min and an exposure duration to smoke containing air of 16 sec per min (14). Cultured cells were exposed for a total of 0, 5, 10, or 15 min to periodic cigarette smoke (Smoke) or clean, filtered air (Air). After the specified exposure period, mediators secreted by exposed cells were collected

immediately from the underlying tissue culture medium. Aliquots (50 μl) were assessed for LDH release, and β–hexoseaminidase activity (500 μl).

cDNA cloning and gene sequencing. Following exposure to Air or Smoke, RBL-J41 cells attached to collagen coated transwells were submerged into growth medium (Dulbecco's Minimal Essential Medium, DMEM) and incubated for a further 1 hour period at 37°C in a standard tissue culture incubator. RNA extraction was carried out on pooled cells from one set of six transwells following each exposure period and this provided sufficient RNA for four RT-PCR reactions using a commercial kit (Stratagene 200345). RNA concentration was determined spectrophotometrically. RT-PCR was carried out on 24 μg RNA according to standard procedures, primers for rat cytokine IL-4 and IL-5 have been described previously (16). The following primer sequences were designed for rat IL-10: 5' GCCAAGCCTTGTCAGAAATG, 3' GTATCCAGAGGGTCTTCAGC; IL-13 5' TATGGAGCGTGGACCTGACA, 3' CCTCAGTGGCCATAGCGGAA; and TNFα: 5' TCCAGAACTCCAGGCGGTGT, 3' TCTTGATGGCGGAGAGGAGG. cDNA synthesis was performed in one thermal cycle (42°C for 50 min, 90°C for 5 min and 4°C for 10min). Following treatment with 1U RNAase H (GIBCO), 0.5μg cDNA was amplified by PCR over 30 cycles (94°C for 1 min, 60°C for 2 min and 72°C for 2 min) employing standard procedures (16). After electrophoretic separation on 2% agarose gels, separated bands were visualized on an UV transilluminator and compared to a 100 base pair (bp) DNA ladder (Pharmacia). Cytokine synthesis in response to an IgE/DNP mediated stimulus was included for comparison. PCR products were excised from the gel (Wizard PCR, Promega) and ligated into pUC 18 ('Ready to Go', Pharmacia) for sequence analysis of the cDNA products.

Results

Figure 1a shows histamine release from dissociated lung tissue cell preparations enriched in HLMC, and 5-HT release from cultured RBL J41 cells, in response to exposure to a cigarette smoke containing atmosphere in a public bar over a period of 4 hours. These mediators are specific indicators of mast cell/basophil degranulation (12,13). Basal levels of histamine/5-HT release from control cells kept in smoke free atmosphere were <4/2.5% respectively of total cellular mediator content and basal release was subtracted from the experimental data shown in Figure 1a. Figure 1b compares β–hex and LDH release from RBL J41 cells maintained at the liquid/air boundary with measurements taken over periods of 0, 5, 10 and 15 min exposure to either Smoke or Air. The effects on the stimulation of cytokine/chemokine synthesis of a 15 min exposure of RBL J41 cells to filtered Air and Smoke, followed by a 1 hour incubation post challenge prior to RNA extraction is shown in Figure 2. Cells exposed to Air, like non-activated cells maintained in a standard tissue culture incubator, express low levels of IL-4, 5, 10 and 13 (Figure 2 lanes 2, 4, 6, and 10). No transcripts for TNFα (Figure 2 lane 8) were detected in cells exposed to Air (Figure 2, lane 11) or cells grown in a tissue culture incubator (data not shown). Following Smoke exposure, we observed distinct increases in transcript levels of IL-4 (350 base pairs [bp]), IL-5 (290 bp), IL-10 (150 bp), IL-13 (298 bp) (Figure 2 lanes 3, 5, 7 and 10), and *de novo* transcription of TNFα (498 bp) (Figure 2 lane 12). Cloning and gene sequencing confirmed the identity ascribed to these bands on the basis of size. Induced synthesis of these cytokines was also observed when cells were triggered via an IgE-mediated antigenic stimulus (data not shown).

Discussion

The present study provides clear evidence that mediators of the allergic response are released from cigarette smoke activated mast cells. The slow release of histamine from HLMC and 5-HT from RBL-J41 cells following exposure to a cigarette smoke containing atmosphere, over a period of 4 hours in a public house shown in Figure 1a, probably reflect cellular responses equivalent to passive smoking. In contrast, data shown in Figure 1b are likely to represent cellular events that occur during active smoke inhalation. Mediator release over a 10 min period, assessed as % β–hex secretion, remains low when cells are exposed to Air (~10%), but rises to ~50% upon exposure to Smoke. An almost four fold increase to 69% is observed by 15 min, when compared to Air (18%). Only modest differences in LDH release (5%) are found between smoke containing and smoke free air after 10 min exposure, while β–hex secretion differs by ~ 40%. Therefore, cytotoxicity alone cannot explain the dramatic increase in β–hex secretion observed in cigarette smoke exposed cells and it is reasonable to conclude that component(s) in Smoke activate intracellular secretion pathways in mast cells.

To evaluate some of the activation events that occur in mast cells in response to Smoke, we assessed differences in cytokine/chemokine expression, since we observed such changes previously in both allergen activated RBL-J41 and HLMC (12,13). Results shown in Figure 2 demonstrate that, when cells are exposed to Air, cytokine/chemokine gene expression remains at a basal level. Exposing cells at the air/liquid interface does not induce TNFα or cytokine gene up-regulation. In contrast, dramatically increased gene expression for IL-4, 5, 10, and 13 and *de novo* synthesis of TNFα can be demonstrated following challenge with Smoke.

The release of inflammatory mediators and the stimulation of the synthesis of pro-inflammatory cytokines by cigarette smoke are highly significant in view of the central role of these cytokines in the genesis and pathophysiological responses associated with allergy and allergic asthma (9,10). They provide explanations for the epidemiological observations that smoking can enhance IgE synthesis since IL-4 and IL-13 induce Ig class switching from IgM to IgE synthesis in B-cells and promote Th2 development (9,10,12,13,16). IL-5, which is chemotactic for eosinophils and activates their growth and differentiation, can account for the chronic pulmonary eosinophilia associated with smoking while IL-10 inhibits Th1 development and stimulates mast cell growth (4-7). TNFα exerts pleiotropic inflammatory effects and contributes to increased vascular permeability, resulting in edema (reviewed in refs. 9,10,16). Particularly significant in this context is the recent demonstration that IL-13 induces asthma through unidentified mechanism(s), which differ(s) from those implicated in classic IgE-mediated allergic responses (9,10).

The molecular mechanism by which cigarette smoke activates mast cells awaits identification. However, preliminary studies have shown that exposure to Smoke, but not to Air, increases protein tyrosine phosphorylation of a range of mast cell proteins (data not shown). The phosphorylation pattern is similar, but not identical, to that observed in response to IgE-mediated degranulation (LJS, University of Sheffield, Ph.D. thesis, 1999).

Active smoking arguably constitutes the largest inhaled oxidant challenge to humans and may be a factor inducing mast cell degranulation and pro-inflammatory cytokine synthesis. Free radicals, oxidants, nicotine and its derivatives have all been identified in the tar and gas phases of cigarette smoke (15). It is particularly relevant to note that oxidative processes can activate

transcription factors of the NF-κB family (17). In man, there is evidence for a linkage on chromosome 5q31.1 between a gene controlling atopy and the IL-4, 5 and IL-13 cluster, whose promoter region has an NF-κB recognition sequence (18). Furthermore, several components in cigarette smoke are chemically related to particles in diesel exhausts, which have been shown to stimulate ongoing IgE synthesis (19), suggesting that related or identical components are responsible for the IgE adjuvant activity of several environmental pollutants.

Data presented in this study provide evidence for the molecular mechanism(s) accounting for the pro-allergenic and asthma-inducing properties of components in cigarette smoke and our conclusions are supported by previous epidemiological observations (1-10). The current study has only focused on the effects of cigarette smoke on cells of mast cell lineage but should be extended to other cells of the airways and their mediators, since it is known that oxidants, including ozone, induce release of e.g. cytokines and mitogenic proteases, by alveolar macrophages and airway epithelia (20). An improved understanding of the molecular mechanism(s) by which environmental pollutants present in cigarette smoke activate the release of pro-allergenic mediators should have important consequences for the design of effective preventative and therapeutic interventions to minimise acute allergic responses. The assay system described in the present study should expedite the identification of components(s) in cigarette smoke responsible for the potential immune modulatory activity, assist its elimation from tobacco or permit the assessment/development of potential antagonists.

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Figure legends

Figure 1 **Comparison of mediator release from human lung tissue enriched in HLMC and RBL-J41 cells.**

Figure 1a: Histamine release from HLMC and 5-HT release from RBL-J41 cells, plated in 24 well tissue culture dishes (Costar), was monitored, after removal of the cover, over a period of 4 hr continuous exposure to cigarette smoke containing air in a public bar. (Bar 1, Sheffield University, ~ 300–350 people present, ~50-60% smokers). Control cells were kept under identical conditions in a cigarette smoke free environment. The ambient temperature in both places was 24°C. Basal histamine/5-HT release (12) from control cells was <4/2.5% during the period under investigation and subtracted from test values. Each point represents the mean (+/- SEM) of data from two and three independent determinations, respectively, carried out in duplicate for HLMC and RBL-J41 cells respectively.

Figure 1b: Beta hex and LDH release from RBL-J41 cells maintained at an air/liquid interface were determined at 5 min intervals over a 15 min period at 37°C to Smoke or Air. Each point represents the mean (+/- SEM) of values from 4 independent determinations, each performed in duplicate.

Figure 1: Mediator (Histamine / 5-HT) and LDH release from cells of mast cell lineage in response to cigarette smoke

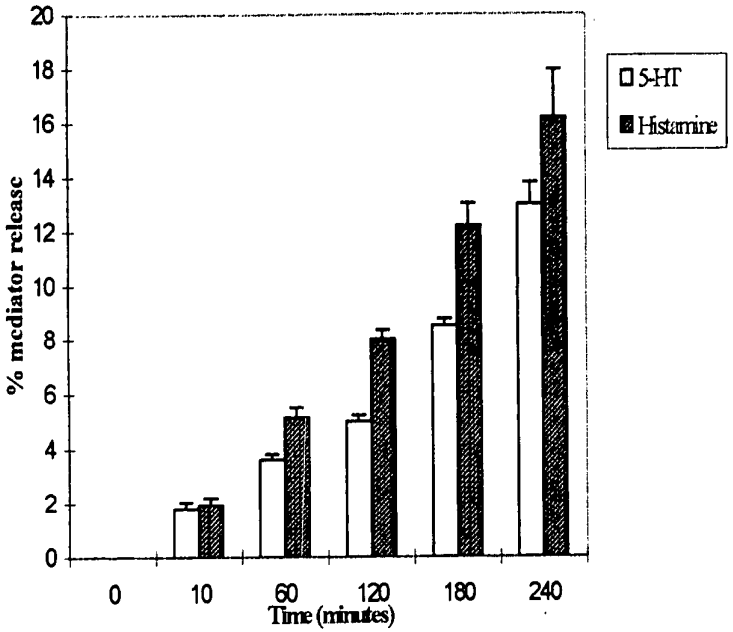


Figure 1 a:
% histamine [■] release from HLMC and 5-hydroxytryptamine [□] from RBL-J41 cells monitored over a period of 4 hours exposure in a cigarette smoke filled room.

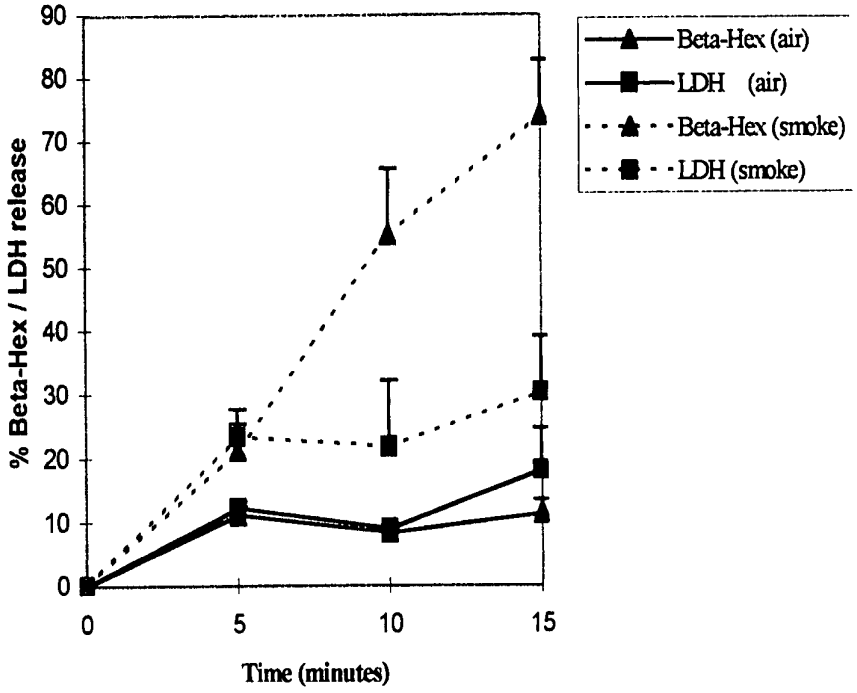
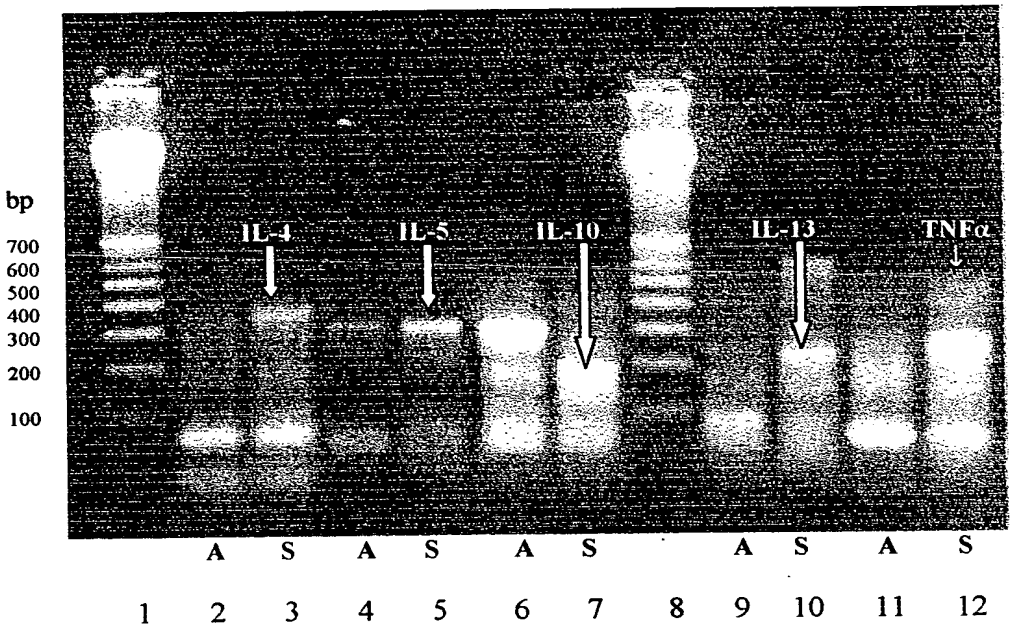


Figure 1 b:
% β-hexoseaminidase [▲] release and LDH [■] from RBL-J41 cells following exposure for 15 min to cigarette smoke (⋯) or clean, filtered air (—) at the cell/air interface.

Figure 2 **Agarose gel showing changes in cytokine/chemokine gene expression in RBL-J41 cells following exposure to clean, filtered Air and cigarette Smoke**.

Cells maintained on a collagen substratum at the air/tissue culture interface were exposed to Smoke (S) (1:100 dilution) or Air (A) at a rate of one inhalation/min and smoke exposure duration of 16 sec per 'puff'. After ten minutes, cells growing on collagen treated transwells were transferred into DMEM and maintained as adhering cells to the collagen substratum for a further 1 hour period at 37°C in a standard tissue culture incubator. RNA isolation, RT-PCR and cDNA synthesis and amplification by PCR were carried out as described in the Methods section. The products were analyzed by agarose gel electrophoresis. Lanes 1 & 8 molecular weight standards, lanes 2,4,6,9,11 cytokine pattern following exposure to Air, lanes 3,5,7,10,12 changes in cytokine pattern following exposure to Smoke. Despite cloning and gene sequencing it was not possible to identify the band at ~300 bp in lane 6, which is prominent in air exposed cells, but disappeared upon smoke exposure.

Figure 2: Cytokine/chemokine gene expression in RBL-J41 cells following exposure to clean, filtered air or cigarette smoke



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Protein and cell engineering of components of the human immunoglobulin E receptor/effector system: applications for therapy and diagnosis

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Abstract. Adaptive immune responses characterised by the synthesis of antibodies of the immunoglobulin E (IgE) isotype play an important role in type I hypersensitivity disorders and parasitic infestations, diseases which have an significant socio-economic impact world-wide. This paper considers potential applications of recent advances in our understanding of the origin of isotype specific immune responses which emerged as a result of cell and protein engineering studies on components of the human IgE/receptor/effector system. Furthermore, the identification of the receptor binding regions in IgE as a result of the development of a stable assay system has important applications for the design of rational therapeutic interventions in allergy and asthma, the treatment of mast cell tumours, and the establishment of procedures for the selective isolation of cells expressing the high-affinity receptor for IgE for functional studies.

Keywords: Immunoglobulin (Ig)E, high-affinity receptor for IgE, allergy, parasitic infestations

1. Introduction

An investigation of structure/function relationships in human (h) immunoglobulin (Ig)E and its receptors together with a study of the molecular mechanisms which cause IgE-mediated hypersensitivity

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reactions and stimulate immunity to parasitic infestations is very timely in view of the socio-economic impact of both diseases. In the developing world some 100 million people suffer from parasitic infestations, while in industrialised countries, the incidence of IgE-mediated allergies and asthma has more than doubled during the past 25 years [1–5].

At birth, IgE levels are either not measurable or exceedingly low. In normal adults, plasma levels of IgE rarely exceed 100 μg/l, but are elevated in allergic and parasitic disease. The sustained production of IgE antibodies in response to parasitic infestations is considered a beneficial immune defence mechanism, while the induction of IgE synthesis by a large number of seemingly diverse and inoccuous antigens is a pathological immune response which results in the development of type I hypersensitivity responses.

An outline of consequences of IgE-mediated target cell activation is shown in Fig. 1. Antibodies of the IgE isotype are usually synthesised and secreted from B lymphocytes in response to allergens or parasite proteins, although substances with suitable adjuvant activity can stimulate an IgE response to by-stander antigens [2,6]. Very little IgE is found in the circulation because IgE antibodies bind with high affinity to Fc receptors (FcεRI) found predominantly on mast cells and basophils, and with low-affinity to receptors (FcεRII) found on various inflammatory cells including macrophages and platelets. At any time, most IgE molecules are cell bound and extensively distributed on the surface of mast cells found in the mucosal lining of the eyes, lungs, skin and the intestine. These IgE sensitised cells are the major target organs in immediate hypersensitivity reactions. Following challenge with cognate antigens/allergens, they respond with the secretion of a wide spectrum of pro-inflammatory molecules, including histamine, prostaglandins, leukotrienes, proteases and chemokines. Cytokines released from IgE-activated mast cells and basophils include interleukin (IL)-4, which induces the expression of MHC class II and FcεRII molecules in target cells. These molecules also provide an up-regulatory feedback signal that stimulates IgE synthesis in B cells. In addition to causing the symptoms of the acute phase of the allergic response, they also induce, via the release of chemotactic mediators such as tumour necrosis factor (TNF) α, the recruitment of inflammatory cell sub-populations, which include eosinophils, macrophages and platelets, into the site of immediate hypersensitiviy, while IL-5 plays a key role in the activation of eosinophils. Eosinophil-mediated cytotoxicity depends on mast cell mediators and in the lungs of asthmatics and tissues invaded by parasites, eosinophils are found in close association with mast cells. Furthermore, oxygen metabolites, which are released from IgE-activated eosinophils, can induce mast cell secretion through an IgE-independent stimulus [6]. These mechanisms contribute to the clinical manifestations of the late phase of the allergic response and illustrate the importance of mediator release from mast cells and basophils in immediate and delayed hypersensitivity responses. An inhibition of the initial degranulation event should therefore be associated with wide ranging short and long term anti-inflammatory benefits [1].

Despite intensive efforts, there are no effective medications to treat allergies and asthma. Currently available therapeutic interventions are inadequate and many are associated with undesirable and often severe side effects. Similarly, attempts to develop effective vaccination schedules for the treatment of parasitic infestations have had only limited success. An improved understanding of the molecular mechanisms involved in these disease processes should assist the development of rational therapeutic interventions.

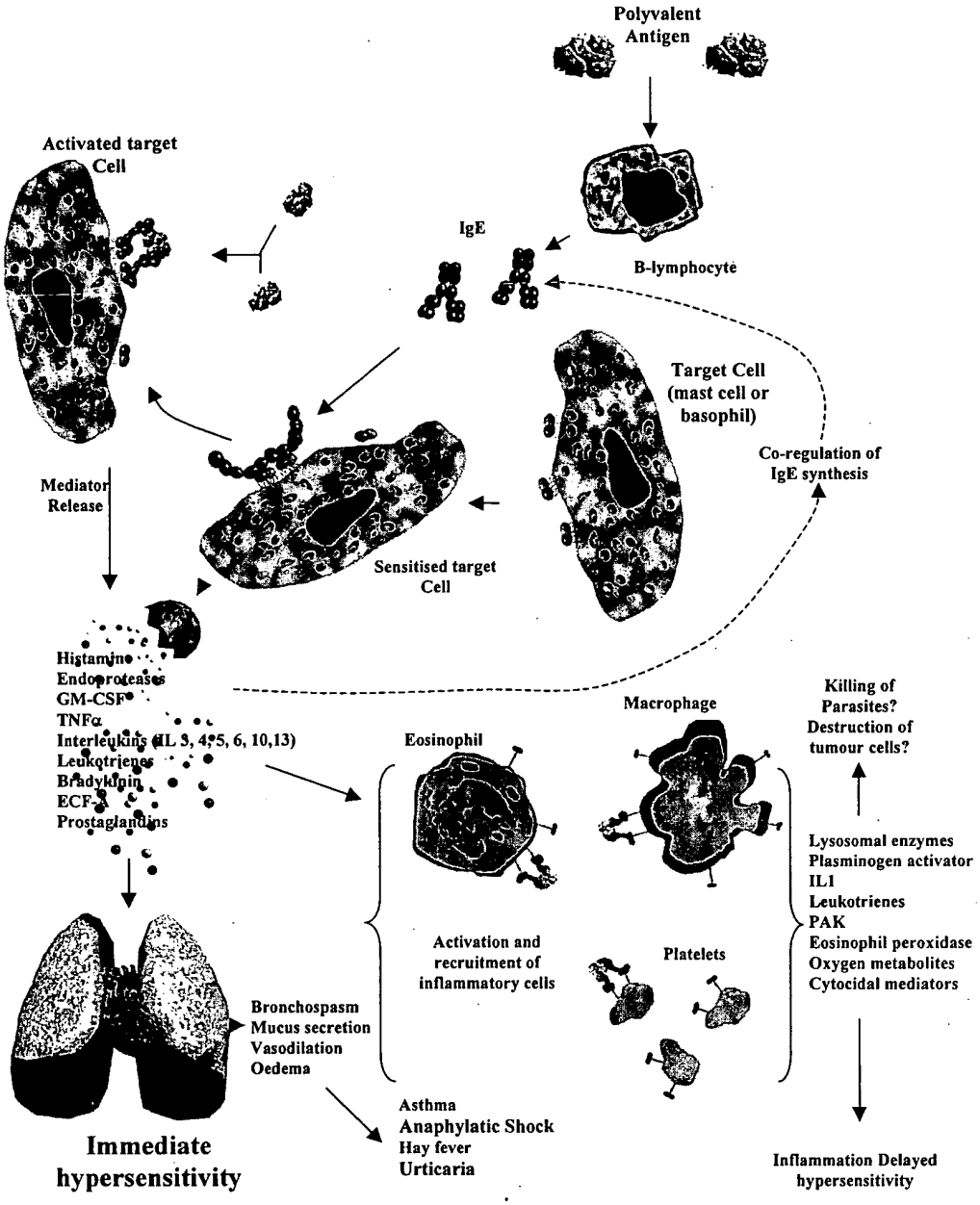


Fig. 1. IgE-mediated cell activation and its consequences in immediate and delayed hypersensitivity reactions. Following synthesis and secretion from B lymphocytes, IgE binds rapidly to high-affinity receptors. The initial interaction does not cause mediator secretion. This take place upon subsequent interaction of receptor bound IgE with cognate antigen. It initiates cell degranulation. Pharmacologically active mediators are rapidly released and these cause the clinical symptoms associated with type I hypersensitivity. In addition, chemokines secreted by IgE-activated mast cells and basophils activate and recruit inflammatory cells into tissues affected by immediate hypersensitivity responses. These cause the symptom associated with delayed hypersensitivity responses. Furthermore, via the secretion of IL-4 an up-regulatory feedback occurs on IgE synthesis by B cells. An inhibition of the initial sensitisation with IgE may therefore be associated with considerable anti-inflammatory benefits. The development of small molecules which block the initial docking of the ligand into the receptor is therefore an important goal of medicinal chemistry programs.

2. Materials and methods

2.1. Generation of rodent cell lines expression the ligand binding domain of the high-affinity receptor for human IgE

Rat basophilic leukaemia cell lines (RBL) expressing the human (h) α-chain of the FcεRI complex were engineered using as a host cell line a high secreting variant of the rat RBL 2H3 cell line [8], which expresses a functional receptor complex for rodent IgE. The h FcεRI α-chain gene was subcloned from pUC19 into the multiple cloning site of the vector pcDNA3 which supports constitutive expression of recombinant proteins in mammalian cells. Correct insertion was confirmed by gene sequencing. The plasmid containing the h FcεRI α-chain gene was transfected by electroporation into the RBL-2H3 cells [8] and is expressed as a functional unit with the rodent receptor on the cell surface. The generation and characterisation of the RBL 2/2/C cell line, which supports dexamethazone inducible expression of h FcεRIα, and the characterisation of IgE binding and secretory responses in native and transfected cells, has been described in earlier publications [8–11].

2.2. Identification of the high- and low-affinity receptor binding site in h IgE

The methodology has been described in earlier publications [1,9].

2.3. IgE-independent activation of mast cell mediators by potential allergens

The methodology has been described in earlier publications [10,11,19,22].

3. Results

3.1. Strategies for the development of therapeutic interventions in allergy and asthma

Although the ancient Egyptians already knew of sudden death as a result of bee stings, the dramatic rise in recent years in the incidence of allergic disease and IgE-mediated asthma in industrialised countries has stimulated the quest for the development of more effective rational therapeutic interventions in allergic disease [1–3].

One such approach focuses on the nature of the complementary binding site between IgE and its receptors. The binding of IgE to both types of receptors is a reversible process which sensitises, but does not induce mediator release until the ligand becomes aggregated, usually by cognate antigen, lectins or anti-IgE antibodies [1,11]. Soon after the discovery of the IgE antibody as the mediator of the allergic response, it was demonstrated that a h IgE fragment, which was prepared by papain cleavage and comprises amino acid (a.a.) residues 227–547 of the disulphide linked Cε2–4 dimer, can competitively inhibit the sensitisation of skin mast cells in passive cutaneous anaphylaxis (PCA) tests [12]. This initiated the search for progressively smaller Fcε peptides as potential blocking agents. In order to overcome the limitations imposed by proteolysis, IgE-derived peptides and chimaeric rodent/human IgE constructs were generated by chemical synthesis or recombinant DNA techniques with the aim of identifying the sequence requirements for the complementary interaction. There is still considerable discrepancy concerning the precise location of the FcεRI binding site in h IgE, although there is now a broad consensus that the binding region for both receptors is located in the Cε3 domain [1]. This disagreement can be

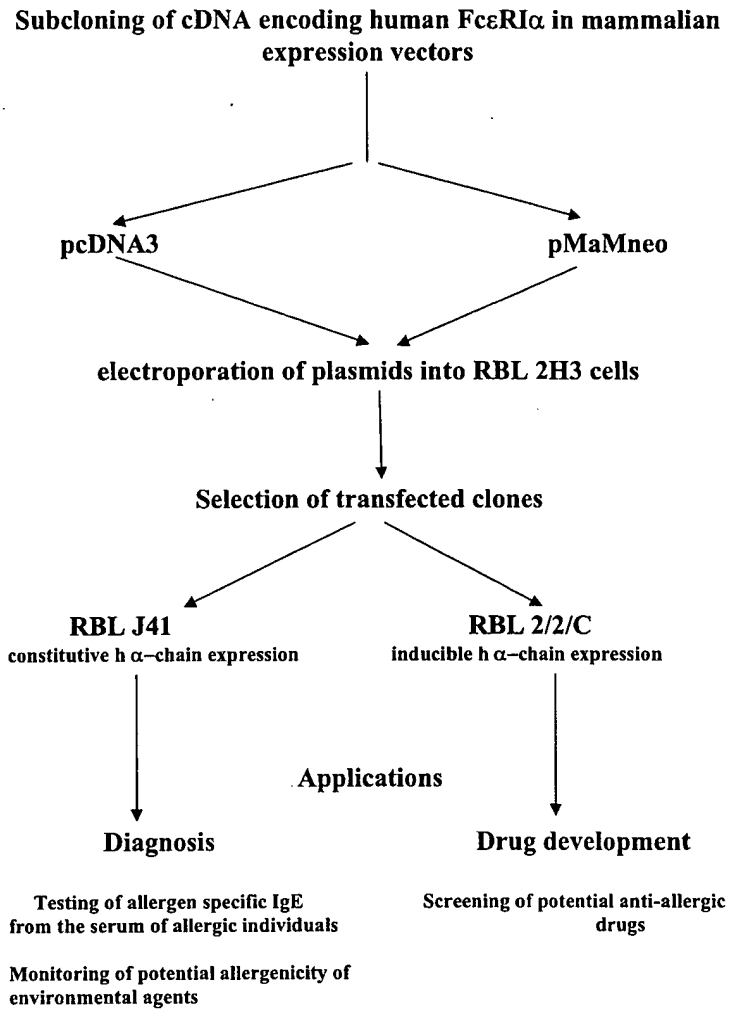


Fig. 2. Establishment of stable rodent mast cell line expressing the ligand binding domain of the human high-affinity receptor complex for IgE. The engineered cell lines support constitutive (RBL J41) and inducible expression (RBL 2/2C) of h FcεRIα. Cells were employed to map the FcεRI binding site in human IgE, using a family of overlapping IgE-derived peptides, shown in Fig. 3. Transfected cells respond to a human IgE-mediated antigenic stimulus with mediator secretion and have useful applications in diagnosis and for the screening of potential anti-allergic drugs.

attributed largely to the fact that h IgE only binds to primate or human FcεRI, and in the absence of a permanent cell line, which expresses the h FcεRI complex, nearly all data reported before the 1990s, relied on the inhibition of the PCA reaction, which produced inconsistent results (reviewed in [1]).

In order to eliminate the problems associated with this temperamental assay system, we developed well-defined *in vitro* assays where the binding of IgE to the soluble extracellular domain of h FcεRIα can be assessed [8,13,14,19]. In addition, we transfected the gene encoding the α-chain of the h FcεRI complex into RBL cells. A functional rodent FcεRI complex is expressed in these cells, which does not bind h IgE. It is made up of an α-subunit, which comprises the IgE binding site, a β-subunit, and two disulphide linked γ-chains. We chose this cell line, which represents an accepted model system for the study of mucosal mast cell function, because earlier investigations had shown that γ-chains of rodent origin can facilitate cell surface expression of h α-chain gene products in, e.g., COS7 cells [15].

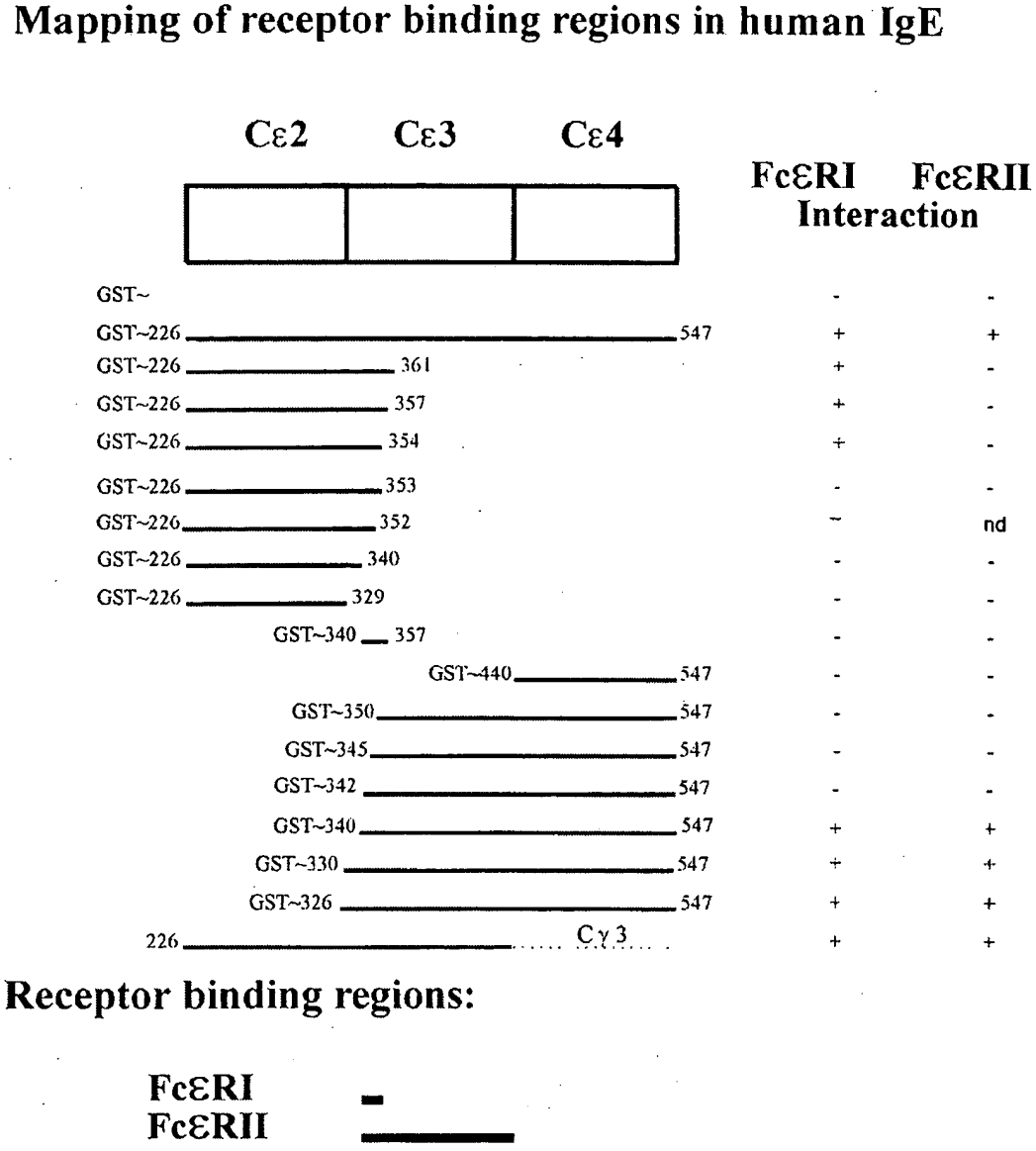


Fig. 3. Identification of the receptor binding regions in human IgE. For experimental details see [1,7].

In addition, the high sequence homology between rodent and human α-chains in the transmembrane domains suggested that the transfected h α-chain should form a functional complex with the β- and γ-subunits of the endogenous rodent receptor complex. Thus, sensitisation with h IgE should activate the host cell's signal transducing machinery in response to a h IgE-mediated antigenic stimulus. Figure 2 shows the technology employed in the engineering of RBL 2/2/C cells, which express $\sim$ 100 000 h α-chains following receptor induction with dexamethazone and that of another variant, RBL J41, which supports constitutive expression of $<$ 10 000 h α-chains per cell. Transfected cells bind h IgE and support h IgE induced mediator release. The RBL 2/2/C cell line was employed to map the FcεRI binding site in h IgE, using a family of overlapping IgE-derived peptides, expressed in *E. coli*, shown in Fig. 3.

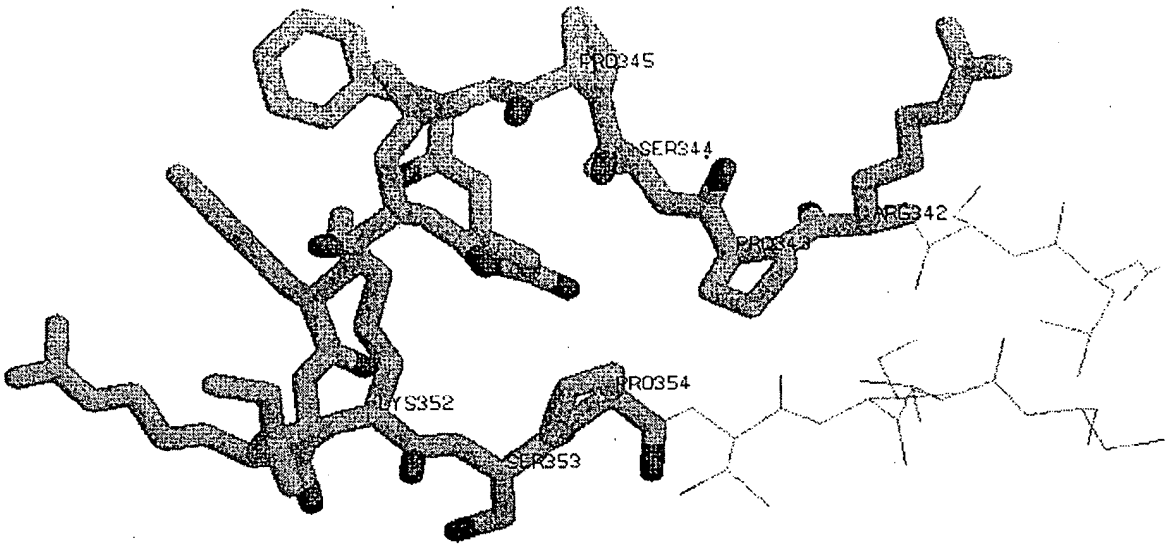


Fig. 4. Model structure of a cyclic peptide based on the A-B loop of h Cε3. The peptide blocks h IgE binding to to h FcεRIα with an affinity in the μmol range and may form the basis for the development of blocking agents which inhibit the binding of IgE to FcεRIα.

Furthermore, values for the kinetics of association and dissociation obtained from the *in vitro* assay system (BiaCore) are in excellent agreement with studies where the binding to the receptor on transfected cells is assessed [14].

3.1.1. Identification of the FcεRI binding site in h IgE: applications for the structure based design of anti-allergic drugs

Figure 3 shows that the sequences common to all Fcε fragments capable of recognising FcεRI comprise Pro343-Ser353 in the Cε3 domain. Further deletion from either the N- or C-terminal end is associated with a loss of receptor recognition [9]. The Pro343-Ser353 peptide blocks IgE/FcεRI binding with an IC_{50} in the mmol range [1,16]. Such low affinity is commonly observed with linear peptides and attributed mainly to the ability of the peptide to adopt a large number of conformations in aqueous solutions. There is however evidence that appropriate conformationally restrained analogues can exhibit enhanced specificity and affinity. Viewed in the context of the model structure we developed for h IgE-Fc, the Pro343-Ser353 sequence has been computed to form an exposed loop [1,9,17], and this provided the basis of the disulphide bond constrained peptide shown in Fig. 4, which blocks IgE/FcεRI interaction in a competitive manner with an IC_{50} in the μmol range. This increase in affinity suggests that this "lead" peptide may form the starting point for the development of low molecular weight anti-allergic drugs [1].

Furthermore, when this peptide was employed to raise antibodies against the FcεRI binding region in h IgE, it was found that these antibodies bind to IgE in solution, but do not recognise receptor bound IgE and inhibit the binding of IgE to FcεRI [1,13]. The structural basis of this phenomenon, which appears paradoxical in view of the fact that IgE is a homodimer and antibodies are divalent, is unknown. It has been explained in terms of a bent conformation of IgE shown in Fig. 5, where the second ε-chain becomes inaccessible to an additional copy of the receptor, or to antibodies directed against epitopes in IgE that become masked following receptor engagement. As our study shows, such IgE epitopes may have applications as immunogens in the therapy of all IgE-mediated allergies through active immunisation irrespective of the nature of the allergen [1].

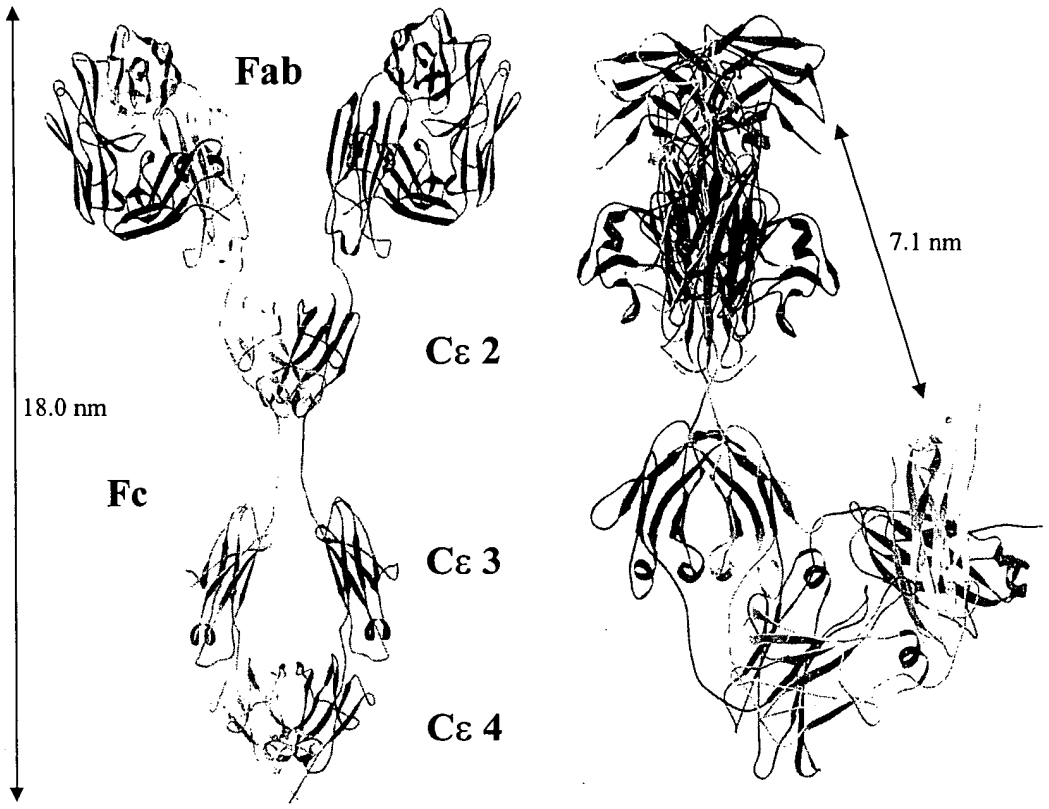
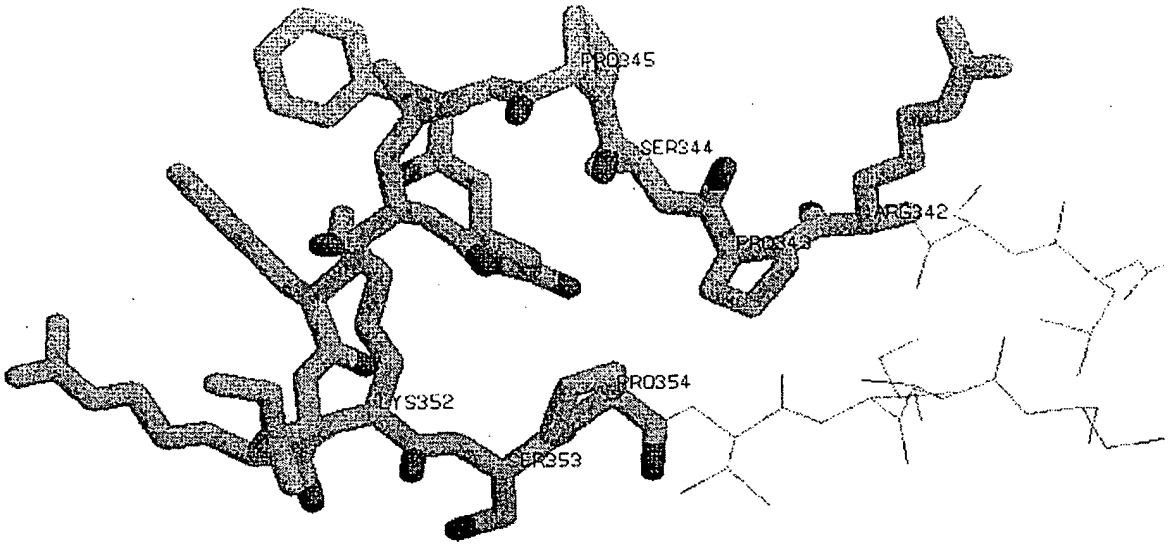


Fig. 5. Structural models of IgE in coplanar (left) and bent conformation (right). A bent conformation of the IgE explains the observation that despite bilateral symmetry, the IgE molecule binds to FcεRIα and non-anaphylactogenic antibodies in a 1 : 1 stoichiometry. It indicates that the identification of epitopes recognised by the receptor of non-anaphylactic antibodies can lead to the design of peptide immunogens for active immunisation of asthmatics and patients at risk of anaphylactic shock.

The development of such vaccines is very timely in view of the dramatic rise in IgE-mediated allergies in recent years for which there exist no obvious underlying cause. Although genetic evidence indicates the importance of hereditary factors in immunity to parasites and susceptibility to develop IgE-mediated allergies, it appears probable that environmental factors play a decisive role in the current epidemic of allergic diseases since the gene pool of the population cannot have changed sufficiently to explain the recent increase in the incidence of type I hypersensitivity responses [2]. Although some observations point to a connection between the decline of infectious diseases and the rise in allergies and asthma [2, 3], there is also compelling evidence that pollutants in air such as diesel exhaust particles, polyaromatic hydrocarbons, oxygen radicals produced by engine emissions or cigarette smoke, can have adjuvant activity and enhance IgE ongoing synthesis [22–29].

3.2. Uncovering a link between the nature of substances that activate cells of mast cell/basophil lineage and the allergic response

The exposure to several types of antigens, including pollen grains, mould spores, house dust mite and cockroach emanations, latex, fruit and nut associated substances or parasite secretions, gives rise with preference to the synthesis of antibodies of the IgE isotype in susceptible individuals. However despite a large amount of information regarding the molecular structure of many allergens and parasite proteins, no



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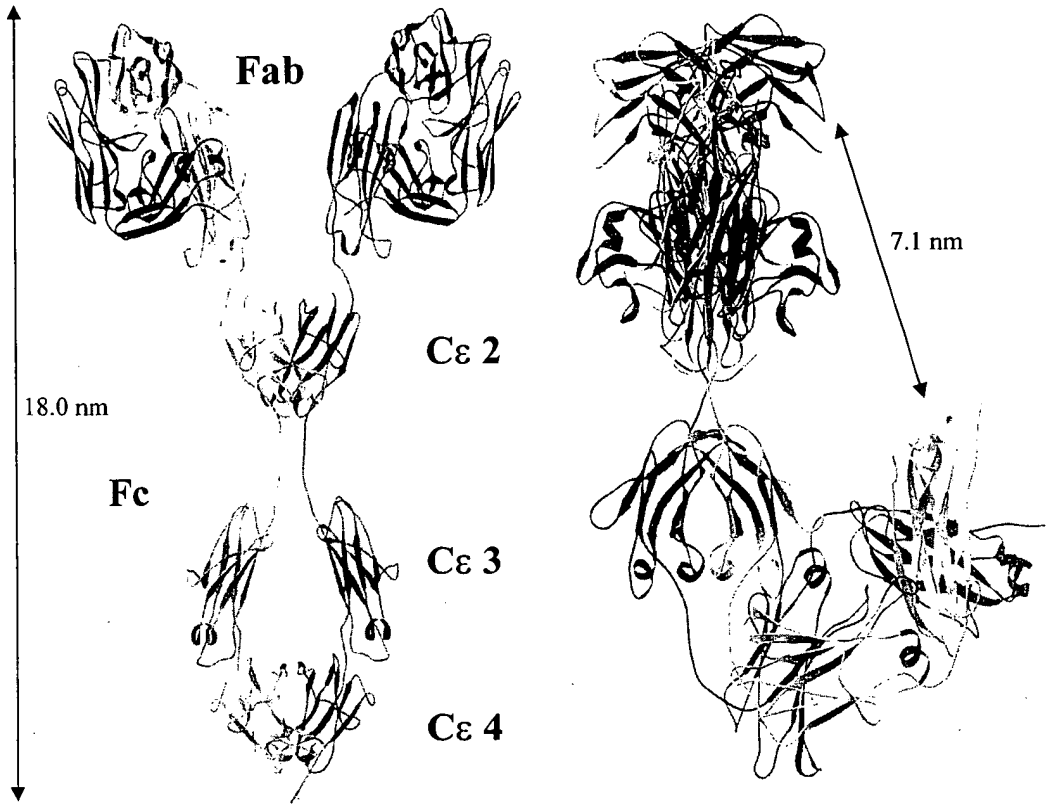

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unifying principle has been proposed that explains the nature of the isotype selection which consistently occurs in response to these diverse substances. Our own studies emerged with an unexpected alternative explanation for the selective isotype induction elicited by these antigens [10,11,18].

This became apparent when we employed the h α-chain transfected RBL cell line to study allergic sensitisation *in situ* as an alternative to the PCA test, since this procedure is associated with the inherent danger of boosting an already sensitised individual [19]. In order to assess the technology, we sensitised the cells with the serum of a bee venom sensitive individual (EMC) and, following challenge with the major bee venom phospholipase A_2 (PLA_2), we could, as expected, demonstrate mediator release. Surprisingly, however, control experiments, where non-sensitised cells had been incubated with the same concentration of antigen in the absence of the serum containing bee venom specific IgE, cells also responded with the degranulation of cellular mediators [10,11,18]. Further studies showed that only enzymatically active bee venom PLA_2, but not an inactive variant, is able to induce IgE-independent mediator release, including IL-4 from this cell line. Furthermore, only mice immunised with enzymatically active PLA_2, but not an inactive variant, produce high levels of PLA_2-specific IgE [10]. This suggested that the catalytic activity, manifested as IgE-independent mast cell secretagogue activity, determined the outcome of isotype specific immune responses.

This initial observation led to an extensive investigation into the IgE-independent activation of cells of mast cell/basophil lineage by potential allergens. Interestingly, potent hydrolytic enzymes, most of which are associated with catabolic pathways, have been isolated from nearly all sources of allergenic materials [20]. Similar proteins are secreted by parasites as part of the invasive process [10,11].

As summarised in Table 1, proteolytic and lipolytic enzymes from organisms as diverse as plants, fungi, house dust mites and schistosomes stimulate degranulation of cellular mediators from RBL cells and induce IL-4 synthesis and secretion. This IgE-independent cell activation is critically dependent on enzymatic activity since inactive forms of, e.g., bee venom PLA_2 or the house dust mite protease Der p I do not induce mediator release [10,11]. Other classes of substances which induce mediators release and stimulate cytokine synthesis are lectins, including those present in natural latex and ragweed, virus associated proteins with protease or lectin-like haemagluttanin activity [11,22], or substances like the polycationic mast cell degranulating agents including mellitin, mastoporan, substance P, and compound 48/80. These are thought to activate heterotrimeric G proteins of the Rab family, which act as regulators of membrane fusion [21]. In addition, components in car engine emissions and cigarette smoke induce mast cell mediator release [23–29]. There is evidence that the former can act as adjuvants, since stimulation of ongoing IgE synthesis has been observed following exposure to diesel exhaust particles and polyaromatic hydrocarbons [23–27] and it is interesting to note that cigarette smoke contains many components also found in diesel engine emissions [24].

Although the RBL cell line presents a cellular model system for the study of mast cell function, we extended our investigation to assess the responses of preparations containing human lung (HLMC) and skin mast cells (HSMC) and basophils to confirm the commonality of our findings. Similar observations were made, indicating that we have identified an important biological principle underlying potential allergenicity [11].

4. Discussion and conclusion

Our results show that protein and cell engineering studies on components of the h IgE receptor/effector system have important applications for the diagnosis and therapy of allergic, parasitic and possibly also viral diseases in relation to the development of allergies [1,10,19].

Table 1

Antigen-induced mast cell mediator release from RBL J41 cells in the absence of sensitisation with antigen specific IgE

	Mediators measured		
	5-HT	Histamine	IL-4
Venoms, bee/wasp (1% suspensions)	90	85	d
Bee venom PLA2 (10 μg/ml) recombinant enzymatically active	17	17	d
Bee venom PLA2 (10 μg/ml) recombinant enzymatically inactive	0	0	nd
Der p I (3 μg/ml) enzymatically active	18	19	d
Der p I (3 μg/ml) enzymatically inactive	0	0	nd
Schistosomal protease (3 μg/ml) enzymatically active	17	19	d
Apergillus protease (10 μg/ml)	21	24	d
Natural latex (1:400) *Hevea brasiliensis*	15	20	d
Condom extract (1:400) (Gossamer)	12	18	d
Hevein (1 μg/ml) *Hevea brasiliensis*	8	9	nd
Influenza virus F (5% suspensions)	5	5	nt
Respiratory syncytial virus (5% suspensions)	7	5	nt

d = detected, n.d. = not detected, n.t. = not tested. 5-HT = [^{3}H]-5-hydroytryptamine (% release). Experimental details have been described in previous publications [10,11]. The enzymatic activity of all enzymes was tested before mediator release was assayed. In the absence of a quantitative assay for rat IL-4, the cytokine was detected by Western blotting.

4.1. Assessment of anti-allergic drugs

The development of stable cell lines, which express the ligand binding domain of h FcεRI and which respond to a h IgE-mediated antigenic stimulus with mediator release, led to the identification of the minimum sequence requirements for the binding to both receptors. This may form the basis of for the design of anti-allergic drugs, based on the structural motif contributed by the A-B loop in the Cε3 domain of h IgE shown in Fig. 4. Both the *in vitro* assay system [14] and the FcεRIa transfected cell lines [9,27] can be employed for the screening and evaluation of potential blocking agents of IgE/receptor interaction and mast cell activation.

The engineering of a variant form of IgE ([IgE] R16) [9,13] which selctively recognises cells expressing the high-affinity receptor, but which does not bind to FcεRII/CD23 has potential therapeutic applications in the treatment of systemic mastocytomas when linked to an immunotoxin or radioactive isotope. In addition, it can be used for the selective isolation of cells expressing FcεRI for functional studies.

4.2. Design of vaccination schedules in allergic and parasitic disease

The demonstration that enzymatically active allergens and parasite proteins activate cells of the immune system to induce cytokine secretion has potential applications for the design of immunisation schedules in allergic and parasitic disease. Immunisation with active parasite protein and/or the use of IL-4 as an adjuvant may induce a protective immune response. Conversely, immunisation schedules employing biologically inactive allergens, anti-IL-4 antibodies, or adjuvants known to induce alternative Ig isotypes may prove effective for the treatment of allergies.

4.3. Development of new diagnostic tests to monitor allergic sensitisation

An *in situ* cellular assay system for the testing of allergic sensitisation offers an attractive and safe alternative to the skin prick test, which may carry the risk of boosting an already sensitised individual [19]. In addition, a considerable number of PCA tests are positive, although no allergen specific IgE can be demonstrated in the patient's serum [19]. This can problably be attributed to the IgE-independent secretagogue which we have shown to be associated with many sources of allergens and our assay system can clearly differentiate between these situations and eliminate potentially false positive results.

4.4. Development of an assay system to predict the potential allergenicity of environmental and occupational hazards

In addition, our observations indicate that the reason why diverse aero-allergens, environmental pollutants, parasite proteins or insect venoms induce and/or enhance IgE synthesis, is their ability to stimulate the release of cellular mediators, including IL-4. It indicates that any substance which induces the secretion of IL-4 must be considered as a potential allergen. The monitoring of air quality involves measurements of NOx gases (nitrogen dioxide, nitric oxide, and sulphur dioxide), ozone, pollen counts and particulate matter. No biological assay exists to assess the immediate and long term effects of these substances on cells of the respiratory tract. Risk assessment by means of a biological assay on cells which closely resemble the mucosal mast cells of the airways [2] is preferable to assessment of epidemiological evidence since this will facilitate pro-active rather than reactive responses when considering airborne allergens in the environment and at the workplace.

The demonstration of non-immunological mediator release from RBL and primary human mast cells and basophils indicate that common cellular responses to these substances occur in rodents and humans. This suggests that RBL cells can be employed to monitor potential allergenicity of occupational and environmental pollutants. The establishment of a cellular assay system to correlate air quality with potential allergenicity may have wide ranging applications in industry and the environment.

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Potential allergens stimulate the release of mediators of the allergic response from cells of mast cell lineage in the absence of sensitization with antigen-specific IgE

A number of structurally diverse antigens preferentially stimulate the synthesis of IgE antibodies, but no unifying principle has been proposed that explains the nature of isotype selection. In the present study, we show that common allergens present in bee venom, house dust mite emanations and parasite proteins induce mast cell and basophil degranulation and stimulate interleukin-4 synthesis, and secretion in the absence of antigen-specific IgE. These data point to a linkage between the initial activation of cells of the innate immune system and subsequent adaptive immune responses. They suggest that IgE-independent mast cell and basophil degranulation is predictive of potential allergenicity and can be evaluated by means of a cellular assay. Our study indicates that non-immunological degranulation by prototypic allergens, such as bee venom phospholipase A_2 or proteases associated with house dust mite emanations, is critically dependent on enzymatic activity. These findings have potentially important implications for vaccine design in allergic and parasitic disease.

1 Introduction

Allergy occurs when the immune system responds to an initial encounter with antigen by producing an antibody of the IgE isotype. Cross-linking of high-affinity receptor (FcεRI)-bound IgE by antigen/allergen results in the release of pharmacologically active agents that cause the clinical symptoms associated with immediate and delayed-hypersensitivity reactions [1–3]. There is evidence that these mechanisms may have evolved to confer protective immunity against pathogens, particularly parasites [2, 4].

A number of environmental factors have been linked to the development of atopic diseases, which affect more than 20% of the human population. It is not known why many diverse and often harmless substances act as allergens or adjuvants to stimulate the synthesis of antibodies of the IgE isotype [3]. In many viral infections, there is an increase in total and virus-specific IgE levels [5, 6]. Despite detailed information regarding the molecular structure of many allergens, a common allergenic principle has not been identified, and the pathogenesis of allergy and asthma still remains to be established.

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Abbreviations: **H:** Histamine **5-HT:** 5-Hydroxytryptamine **h:** Human **IgE-VNP:** NIP-specific IgE **FcεRI α:** Ligand binding domain of the high-affinity receptor for hIgE *Der p: Dermatophagoides pteronyssinus* **HDM:** House dust mite **SGM:** Spent growth medium **NIP-HSA:** 4-hydroxy-3-nitrophenyl-acetyl coupled to human serum albumin **LDH:** Lactate dehydrogenase **SE:** Staphylococcal enterotoxin **HLMC:** Human lung mast cells **HSMC:** Human skin mast cells **ASA:** Acetylsalicylic acid

Key words: Allergen / Mast cell activation / Interleukin-4

The molecular mechanisms controlling antibody isotype selection following encounter with a given antigen are unclear. The regulatory role of cytokines is established, and interleukin (IL)-4 has a central role in the induction of IgE antibody responses. Eosinophils, mast cells, and basophils have been shown to be a source of IL-4, in addition to T lymphocytes [7–12]. It has been proposed that the interaction of helminths or allergens with these cells induces IL-4 release. The only known physiological stimulus inducing IL-4 production by these cells is the cross-linking of FcεRI, but this could occur only after specific IgE antibody responses are established [13]. Following the engineering of the RBL-2/2/C cell line (RBL-2H3.1 cells [14] transfected with the α subunit of the human FcεRI [15, 16]), we observed IgE-independent mediator release when cells were challenged with allergens, including house dust mite emanations and bee venom, prior to sensitization with antigen-specific IgE [3, 17]. Enzymatically active bee venom allergen phospholipase (PL)A_2, but not an inactive variant, is able to induce IgE-independent mediator release, including IL-4 from this cell line [17], and it is only when mice are immunized with active PLA_2 that high levels of PLA_2-specific IgE occur [17]. Since IL-4 directs T helper cell differentiation [13] and B cell class switching to both IgG and IgE, we suggested that cells of mast cell, basophil, or both lineages might be the initial source of IL-4 and that interaction of allergens or pathogens at mucosal surfaces will generate a stimulus capable of steering subsequent adaptive immune responses [17].

Evidence indicates that the action of antigens on components of the innate immune system determines subsequent adaptive responses [18]. Following infection with *Listeria monocytogenes* [19], macrophages become a source of IL-12 which induces Th1 development of naive $CD4^+$ T cells. $FcεRI^+$ cells are the major source of IL-4 following *Schistosoma mansoni* infestation [20]. The protective role of mast cells in bacterial infections has just become apparent [21–24]. Although the moleular basis for cell activation remains to be established, we have already proposed that a number of allergenic and parasitic proteins stimulate

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secretion of mediators of the allergic response independently of IgE sensitization, and that this action precedes subsequent IgE responses [3, 17]. We have examined this hypothesis further by extending our studies to a number of potential allergens and assessed IgE-dependent and independent induction of mediator release from RBL-2/2/C cells [15], from human leukocyte preparations enriched in basophils, and from human lung and skin mast cells. We demonstrate the non-immunological release of mediators of the allergic response following challenge with aspirin-based drugs, lectins, lipopolysaccharides, endotoxins, viral particles, allergenic, and parasite antigens, such as bee venom, house dust mite emanations and parasite secretions which are rich in hydrolytic enzymes.

2 Materials and methods

2.1 Source, isolation and characterization of antigens

Recombinant mite protease *Dermatophagoides pteronyssinus* (*Der p*)I [25] was provided by Dr. T. Merrett, Allergy Analysis Centre, Euro/DPC, Ltd., Glyn Rhonwy, Llanberis, GB. House dust mite (HDM) extracts from mites of the genus *Dermatophagoides* (D1912, 4001, 4002, 4020) were purchased from SmithKline Beecham Pharmaceuticals, GB. Despite a low proteolytic activity, these preparations retain allergenic epitopes. HDM colonies (gift of Dr. M. Colloff, University of Glasgow, GB) were maintained in 9-cm petri dishes at 75% relative humidity at room temperature, fed with baker's yeast and subcultured weekly by replacement of two-thirds of the dish contents with fresh yeast. For large-scale preparation of crude extracts containing mite emanations, spent growth medium (SGM) from several mite cultures was pooled and separated from growth medium (baker's yeast) by passage through a sieve. SGM, mite debris and feces were resuspended in 2 ml PBS following homogenization, and extracts were kept at −20°C until further use. Proteolytic activity associated with HDM emanations was isolated from 200 mg freeze-dried SGM which was taken up in 4 ml PBS and loaded onto a Sephadex G200 column equilibrated with the same buffer for size exclusion chromatography (Pharmacia LKB Biotechnology). Fractions in the regions corresponding to 25 and 30 kDa were pooled and concentrated by ultrafiltration using a YM-10 membrane (Amicon). Concentrated pooled samples were reloaded onto a Superdex-75 column for separation of the 30 and 25 kDa components. In each instance SDS-PAGE analysis [26] of the peak fractions showed the presence of one major (>95%) and several minor bands. The major bands were recognized in immunoblots probed with serum (1:10 dilution) from a skin test-positive HDM-allergic individual (T.A.). Blots were developed with a horseradish peroxidase-labeled rabbit anti-human IgE antibody (P0295, Dako, GB) [27].

For the isolation of cercarial proteases, a Puerto Rican isolate of *Schistosoma mansoni* was maintained by routine passage through LACA mice and *Biomphalaria glabrata*. Snails harboring a patent infection were induced to shed cercariae, which were concentrated by sedimentation at 4°C [28]. Resulting schistosomula were cultured for 3 h in 50-ml flasks at 37°C in medium 169 in 6% CO_2 in air. The parasite suspension was then centrifuged at 150 × g for 90 s and the culture supernatant removed for concentration by ultrafiltration with a 3-kDa-cutoff Diaflo membrane (Amicon) under N_2 pressure (40 psi). SDS-PAGE [26] analysis revealed the presence of several bands. Serine protease activity was shown to be associated with bands in the 20–25-kDa region.

The catalytic activity of proteolytic enzymes was assayed using Azocoll (A9409), tosyl sulfonyl L-arginine methyl ester (TAME, T4626) and benzoyl-L-arginine ester hydrochloride (BAPNA, B1007) as substrates [29], all purchased from Sigma. Cysteine proteases were activated in buffer containing 0.01 M EDTA, 0.05 mM 2-mercaptoethanol, and 5 mM cysteine, and passed through a Sephadex G-20 spun column immediately before use. Overnight storage of enzyme preparations at room temperature resulted in the almost complete loss of proteolytic activity (>99%) due to autoproteolysis, as evidenced by SDS-PAGE analysis (data not shown).

All other proteases, lectins and staphylococcal enterotoxins were purchased from Sigma (see Table 1). Dispersible aspirin (Mandaprin) was purchased from M&A Pharmachem, Bolton, GB.

Influenza virus strains Hong Kong (H_3N_2) containing 270 μg hemagglutinin/ml, Texas-like strain containing 520 μg hemagglutinin/ml, X1AC, F and ALUSSR (H_1N_1), herpes virus and equinus flu virus were provided by Prof. R. Jennings (Department of Experimental and Clinical Microbiology, University of Sheffield, GB), and purified influenza virus hemagglutinin was from Prof. J. J. Skehel (MRC, Mill Hill, London, GB).

2.2 Cell culture and measurement of mediator secretion with or without sensitization with antigen-specific IgE

Mediator release from RBL-2/2/C has been described in earlier studies [15–17, 30] and human (h)FcεIα chain expression is not required to assess IgE-independent mediator release. Cell culture procedures and measurement of 5-hydroxytryptamine (5-HT) and β-hexoseaminidase secretion, and IL-4 and MHC class II up-regulation, have been described [16, 17]. The percentage of histamine released by RBL-2/2/C was evaluated by measurement of histamine in 50 μl cell supernatant using an ELISA kit (Miles Inc., CT).

2.3 Lactate dehydrogenase (LDH) assay

Antigen cytotoxicity was determined using a nonradioactive cytotoxicity assay (CytoTox 96, G1780, Promega, Southampton, GB) to measure the activity LDH in 50 μl cell supernatant containing antigen-induced mediators.

2.4 Preparation of human lung and skin mast cell suspensions

Cell suspensions containing human lung mast cells (HLMC) and human skin (foreskin) mast cells (HSMC) were prepared by physical and enzymatic dispersion [31].

Table 2. Antigen-induced release of mediators of the allergic response from RBL-2/2/C cells without IgE sensitization[a]

Antigen	Mediators measured 5-HT	Histamine	IL-4[b]	LDH
Bee venom (1 μg/ml)	87.0 ± 8.4	85.0 ± 3.2	d	82.0
Bee venom PLA_2 (10 μg/ml)	16.7 ± 1.0	7.3 ± 0.7	d	16.0
Phospholipase C (*Bacillus cereus*, 10 μg/ml)	46.4 ± 8.8	33.0 ± 9.6	n.t.	27.0
Thrombin (10 μg/ml)	6.4 ± 2.5	n.t.	n.t.	n.t.
Trypsin (10 μg/ml)	18.9 ± 2.7	n.t.	d	0
Papain (10 μg/ml)	17.0 ± 2.6	21.2 ± 3.2	n.t.	n.t.
HDM-SGM inactive (10 μg/ml)	0	0	n.d.	n.t.
HDM-SGM active (10 μg/ml)	26.5 ± 10.6	26.0 ± 5.5	d	n.t.
Der p I (cloned, 3 μg/ml)	0	n.d.	n.t.	n.t.
Schistosomal protease (3 μg/ml)	18.5 ± 1.7	19.7 ± 2.3	d.	0
Aspergillus protease (100 μg/ml)	24.5 ± 0.8	33.0 ± 7.1	d.	0

a) Enzymatic activity of all proteolytic enzymes was assayed immediately before use. The concentration of active enzyme is based on substrate hydrolysis and that of inactive enzyme is based on protein concentration. The percentage of mediators (5-HT, Histamine) and LDH secreted by RBL-2/2/C cells after 15 min of antigen stimulation was calculated as described [28]. The values show the mean and SE of at least three tests performed in duplicate. Similar observations were made with other proteases listed in Sect. 2.1 (data not shown).

b) In the absence of a quantitative assay for rat IL-4, the cytokine was detected by Western blotting: d = detected and n.d. = not detected, n.t. = not tested.

been attributed to the cytolytic peptide melittin [44]. Release of mast cell mediators *in vivo* following a bee sting therefore involves a cytolytic route, which is nevertheless of physiological importances since human mast cells and basophils contain preformed mediators which will be set free following cell lysis [8–12].

In contrast, SGM containing enzymatically active HDM proteases and purified *Der p* I stimulate mediator secretion by a noncytotoxic mechanism at low enzyme concentrations (0.5–50 μg/ml) with cytotoxic release at concentrations higher than 100 μg/ml. This dose-dependent effect has been observed for a number of serine proteases, including trypsin, which is commonly used to release RBL cells from plastic surfaces before studying the physiological consequences of IgE-mediated receptor activation in suspension cultures [50]. Our study indicates that the results of such experiments require re-evaluation.

The molecular mechanism by which proteases induce mediator release by a noncytotoxic mechanism remains to be determined. We have previously suggested that cleavage of the extracellular domain of the FcεRI α chain occurs at protease sensitive sites (residues 170–179) in close proximity to the cell membrane [43]. Receptor cleavage has also been reported for the extracellular portion of human FcεRII/CD23 by *Der p* I [51, 52]. Truncated receptors may aggregate due to removal of extracellular constraints, resulting in the generation of an activated receptor complex and ultimately degranulation.

Further evidence for a mechanism of activation by extracellular domain cleavage was obtained when we studied the dose-response relationship between *Der p* I protein concentration and 5-HT release from RBL-2/2/C cells. Fig. 1a shows the percentage of 5-HT released by RBL-2/2/C cells triggered with catalytically active *Der p* I with or without sensitization with IgE from an HDM-sensitive individual (T.A.). A bell-shaped dose-response pattern, characteristic of receptor clustering, is observed in both cases. Comparable results (data not shown) were obtained when unsensitized cells were incubated with trypsin or papain. In contrast, as shown in Fig. 1b, inactive degraded *Der p* I possessing less than 1 % of its original activity causes degranulation only when cells are sensitized with IgE from an HDM-sensitive individual, indicating the persistence of allergenic epitopes in the degraded allergen. Similar observations were made when cells were incubated with catalytically inert recombinant *Der p* I protein (data not shown) [48].

3.2 IL-4 metabolism in antigen-stimulated RBL-2/2/C cells

The release of IL-4 and increased synthesis in response to bee venom and *Der p* I can be demonstrated by Western blot analysis [17] as shown in Fig. 2A, panels a and b. It is observed within 15 min of challenge and peaks between 2–4 h after the initial stimulus. The kinetic pattern is similar to that observed following an IgE-mediated antigenic stimulus (Fig. 2B). Several anti-IL-4-reactive polypeptide bands are detected in Western blots of protein extracts from the cell pellets of stimulated RBL-2/2/C. We [17] and others [53] have made this observation previously. A FAST search of the SWISS PROT data base revealed no significant sequence homology between IL-4 and other known proteins, so we attributed these bands to the presence of different glycosylated forms of IL-4 and pro-IL-4 [17, 53], although the expression of alternatively spliced forms of IL-4 cannot be ruled out [54].

3.3 Up-regulation of class II/Ia major histocompatibility antigens

IL-4 induces the up-regulation of a number of membrane proteins, including FcεRII and MHC class II/Ia antigens.

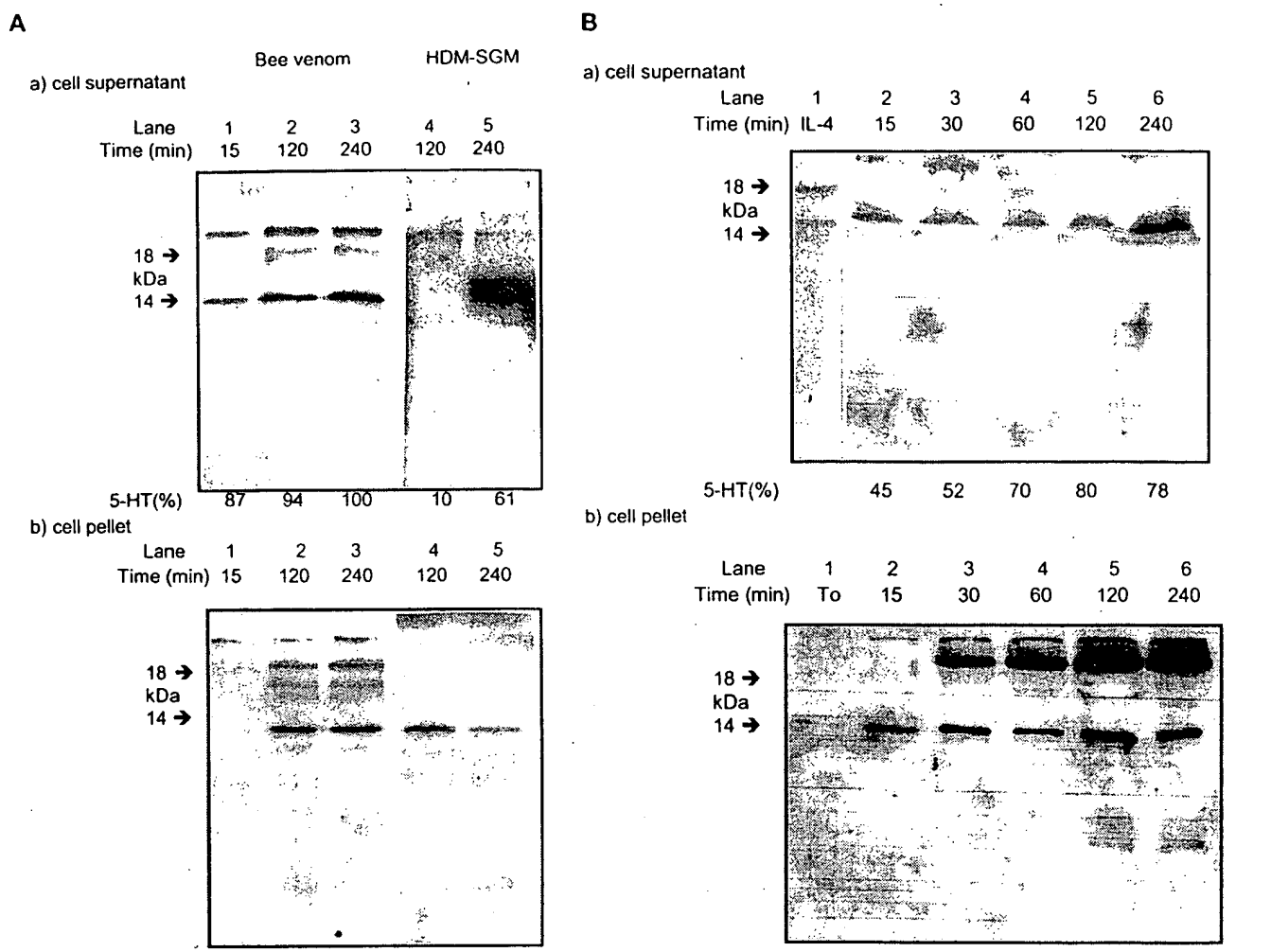

Figure 2. (A) The kinetics of 5-HT secretion and IL-4 synthesis and secretion in response to challenge with bee venom and spent growth medium from house dust mites is shown. RBL-2/2/C cells were cultured for 24 h in the presence [^{3}H]5-HT. Cells were washed twice before the addition of a bee venom suspension and HDM-SGM. Lanes 1–3 = bee venom suspension (1:1000), lanes 4–5 = house dust mite SGM (3 μg/ml). Panel (a) shows the percentage of 5-HT secretion and immunoreactive IL-4 proteins, detected by Western blotting, secreted into the culture medium over a 4-h period; panel (b) IL-4 in cell pellets. (B) The kinetics of 5-HT secretion and IL-4 synthesis and secretion in response to an IgE-mediated antigenic challenge is shown. RBL-2/2/C cells were incubated for 24 h with NIP-specific IgE (1 μg/ml) followed by challenge with 100 ng/ml NIP-HSA [16]. 5-HT and IL-4 secretion (panel a) and levels of IL-4 in cells pellets (panel b) were monitored over a period of 4 h. Panel (a): lane 1, positive control for anti-rat IL-4-reactive proteins secreted by CHO cells [53]; panel (b): lane 1, total cellular IL-4 content in RBL cells prior antigen stimulation; lanes 2–6, IL-4 in supernatants (a) and pellets (b) after antigen stimulation.

The increased expression of these molecules on rat B splenocytes in response to supernatants obtained from *Der p* I and schistosomal protease-triggered cells is shown in Fig. 3. It provides further support that the supernatant from RBL-2/2/C cells following non-immunological stimulation contain a factor which is capable of inducing a response similar to that caused by IL-4. The demonstration of increased expression of MHC class II/Ia antigens is important, since the quantity of antigen displayed by the processing cell is related to the density of these molecules.

3.4 Mediator release from human mast cells and basophils

Although the RBL-2H3.1 cell line represents a model system for the study of mast cell function [14], responses to a number of stimuli may differ from those observed with HSMC, HLMC, and basophils.

Table 3 summarizes the effect of bee venom PLA_2, HDM and schistosomal proteases on dissociated cell preparations of human lung and skin tissue, and partially purified HLMC and HSMC and basophils. Histamine release, which is a specific indicator of mast cell/basophil degranulation, occurs in response to catalytically active HDM, schistosomal proteases and PLA_2, but not inactive antigens. Unlike mast cells, basophils do not respond to a PLA_2-mediated stimulus. We have already demonstrated that the enzymatically inactive PLA_2H34Q does not degranulate cells unless they are sensitized with IgE [17] and, as shown in Table 3, HLMC challenged with commercial HDM-SGM (4001), which retains only low levels of protease activity, release basal levels of histamine. It shows that preparations containing HLMC release histamine solely in response to enzymatically active *Der p* I and that degranulation cannot be attributed to cell triggering by allergen-specific IgE. We detect IL-4 secretion from mast cells after exposure to PLA_2 and proteases, and the latter

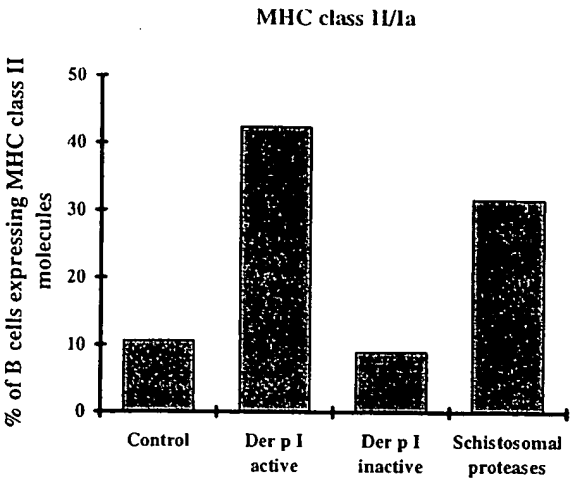

Figure 3. Up-regulation of MHC class II expression on rat B splenocytes by supernatants from RBL-2/2/C cells triggered with *Der p* I and schistosomal proteases. Rat splenic B cells were isolated by immunomagnetic depletion of splenic T cells as described [17], and incubated at 37 °C for 24 h with culture medium (control) or supernatants from RBL-2/2/C cells, triggered with 3 μg/ml of active and inactive *Der p* I and schistosomal proteases without sensitization with antigen-specific IgE. The expression of MHC class II/Ia antigens was analyzed by flow cytometry using an anti-MHC class II/Ia FITC-labeled antibody [17]. Each bar represents the mean of three independent experiments carried out in duplicate.

also stimulate IL-4 secretion from preparations enriched in basophils. Since these preparations are impure, we cannot be certain that the source of IL-4 is the allergen-triggered mast cell. The demonstration of IL-4 generation from human tissues in response to an antigenic stimulus must in any case be considered significant in view of the pivotal role of this cytokine in steering adaptive immune responses. However, we found that the amount of IL-4 secretion based on mast cell content is similar for dissociated lung tissue and enriched preparations of mast cells and that this is not significantly influenced by purification procedures [33, 34]. Two HLMC preparations containing 50 and 81 % Alcian blue-stained mast cells, challenged with 3 μg/ml bee venom PLA_2 and schistosomal protease, released 300 and 120 pg of IL-4 per 10^6 Alcian blue-stained cells, respectively.

4 Concluding remarks

Matzinger [55] has recently proposed that acquired immunity depends on the ability of the immune system to recognize danger. Based on our current and previous studies [17], we suggest that the outcome of adaptive immune responses is dependent on the interaction of antigens with cells of the innate immune system. Our observations suggest that the reasons why diverse aero-allergens, insect venoms and invading parasites stimulate IgE synthesis is related to their ability to induce the release of cellular mediators, including IL-4. Endogenous mast cell proteases and PLA_2, which are secreted from triggered mast cells or basophils, have also been shown to activate cellular functions in diverse cells [56–61], while prostaglandin E_2 synergizes with IL-4 and LPS to induce IgG1 and IgE production [62]. This indicates that the initial stimulation of cells by exogenous antigens can be amplified through the release of endogenous mediators. Our demonstration that many antigens induce cells of the innate immune system to release IL-4 has important implications. In combination with this cytokine, mast cells and basophils are able to furnish, via CD40L, the minimal essential signals necessary to induce class-switching to IgE in B cells [63]. It suggests that antigen-activated cells can play a crucial role in the induction of Ig class switching. *In vivo,* where a cock-

Table 3. Release of histamine and IL-4 from preparations containing human lung (HLMC) and skin (HSMC) mast cells, and from basophil enriched leukocyte preparations stimulated with bee venom phospholipase A_2, house dust mite and schistosomal proteases[a)]

Antigen	HLMC				HSMC				Basophils			
	Histamine (%)		IL-4 (pg/10^6 cells)		Histamine (%)		IL-4 (pg/10^6 cells)		Histamine (%)		IL-4 (pg/10^6 cells)	
	n		*n*		*n*		*n*		*n*		*n*	
Bee venom PLA_2 (3 μg/ml)	7	20.4 ± 5.7	4	168 ± 26.1	6	26.9 ± 18.3	1	6	3	0		n.t.
HDM-SGM (10 μg/ml)	4	15.3 ± 4.6	2	23 ± 8.4	7	29.1 ± 16.1	1	3	2	13.7 ± 3.6	2	52 ± 8.0
HDM-SGM (4001) (10 μg/ml)	2	3.2 ± 2.2		n.t.		n.t.		n.t.		n.t.		n.t.
Der p I (10 μg/ml)	4	13.6 ± 4.0	2	65.4 ± 0.8	5	28.7 ± 11.4	1	5	1	9.6		n.t.
Der p III (10 μg/ml)	4	12.4 ± 2.9	2	102 ± 3.3	7	13.1 ± 6.3	1	4	1	2.8		n.t.
Schistosomal protease (10 μg/ml)	3	23.2 ± 8.5	3	35 ± 18.7	6	18.4 ± 7.7	1	0	3	21.7 ± 9.8	2	39 ± 8.7

a) Experiments on preparations containing 1×10^6–2×10^6 cells were performed in duplicate. The proportion of Alcian blue-stained cells in cell preparations was: HLMC = 10–20 %, HSMC = 2–5 %, basophils = 3–10 %. Results for histamine and IL-4 are presented as the mean value ± SE; n = number of experiments performed in duplicate. The release was corrected for background and is expressed relative to total cellular histamine content. IL-4 values were corrected for spontaneous release (<3 pg/10^6 cells). No histamine or IL-4 release was detected after cell challenge with enzymatically inactive proteases and PLA_2.

tail of antigens is usually encountered, substances which stimulate IL-4 secretion may act as IgE-specific adjuvants and induce an IgE response to bystander antigens [64]. Any substance which stimulates synthesis and secretion of IL-4 must therefore be considered as potentially allergenic. Non-immunological mediator release from RBL-2/2/C and human cells indicates that similar mechanisms operate in rodents and man, and suggests that RBL cells may be used to evaluate potential allergenicity.

The demonstration that enzymatically active allergens and parasite proteins activate cytokine secretion has important implications for the design of anti-parasite and anti-allergy vaccines. Immunization with active parasite protein or the use of IL-4 as an adjuvant may lead to a protective immune response. In contrast, for the treatment of allergies, immunization with biologically inactive antigens may stimulate an IgG response as demonstrated in our previous investigation [17].

The current study has concentrated on the role of antigens in activating cells of mast cell/basophil lineage. The action of phospholipases and proteases on the stimulation of metabolic pathways, which has been described for several different cell types [43, 44, 49, 56–60, 65], can be expected to modulate a variety of responses *in vivo*. The cleavage of FcεRII/CD23 by *Der p* I from cells of B cell lineage [51, 52] is a pertinent example which demonstrates that the encounter with a potential allergen can affect diverse cells in a physiologically significant way. Complement activation by protease antigens deserves attention in view of the fact that components of the complement cascade are activated by proteolytic cleavage [42] and complement receptors are found on many cells of the immune system, including mast cells and basophils.

The mode of activation of cellular responses and the route by which mediator secretion and induction of IL-4 and possibly that of other cytokines occurs, remains to be established. The identification of these pathways may provide important information for our understanding of the stimulation of basic immune mechanisms.

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A Link Between Catalytic Activity, IgE-Independent Mast Cell Activation, and Allergenicity of Bee Venom Phospholipase A_2[1]

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The molecular and cellular mechanisms controlling Ab isotype selection following encounter of a given Ag are still unclear, although the regulatory role of cytokines is established. In the present study we explored the possibility that the nonimmunologic interaction of an allergen with cells of the innate immune system might result in a release of mediators that promote IgE isotype selection in adaptive responses. Using the bee venom allergen phospholipase A_2 (PLA_2) and a mutant variant lacking enzymatic function, we show that PLA_2, but not its catalytically inactive variant, is able to induce IgE-independent mediator release, including IL-4, from rodent mast cells. Assessing the in vivo relevance of these observations, we find that repeated injections of low doses of active enzyme into mice induce the synthesis of high levels of PLA_2-specific IgE, while immunization with the inactive form yields no detectable IgE response. Both Ags were similarly immunogenic when high doses of Ag were used for immunization. These findings suggest that mast cells might be a source of IL-4 at the onset of specific immunity against sources of allergens such as bee venom that contain PLA_2 and support the concept that the biologic action of an Ag on cells of the innate immune system can play a key role in determining adaptive immune responses. *The Journal of Immunology,* 1995, 155: 2605–2613.

In mammals, the exposure to certain Ags gives rise to a preferential synthesis of IgE Abs, suggesting that the isotype selection may be related to the nature of the Ag. The sustained production of IgE Abs as a result of parasitic infestations is a well recognized and beneficial immune defense mechanism (1). In contrast, the induction of IgE synthesis by a large number of seemingly diverse and mainly innocuous Ags, including pollen grains, mold spores, house dust mite and cockroach emanations, insect venoms, and food components, is a pathologic immune response linked to the development of allergy and asthma (2). The reasons why these Ags cause abundant IgE Ab synthesis are poorly understood. The possibility that certain Ags share common structural motifs that selectively induce class switching to IgE has been investigated extensively, but despite intensive efforts and a considerable amount of information regarding the molecular structure of many common allergens, no unifying feature has emerged (2).

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In humans as well as in rodents, it is established that an elevated IgE Ab synthesis is associated with a Th cell response predominated by cytokine profiles of the Th2 phenotype (3, 4). The current concept is that Th2 and Th1 subsets differentiate from a common precursor, which has the capacity to secrete Th1- and Th2-type cytokines (5). Recent in vitro and in vivo studies have emphasized the pivotal importance of the cytokine environment present during the priming of these naive precursor T cells in determining their preferential differentiation into Th1 or Th2 subsets: IFN-γ and IL-12 drive Th1 differentiation, whereas IL-4 promotes Th2 development (6–8). This raises questions about the molecular and cellular mechanisms by which an Ag or invading pathogen can create such a cytokine environment at a time when specific lymphocyte responses are not yet established (5, 9).

Regarding the mechanisms leading to preferential Th1 responses following infections with intracellular parasites, it has been shown that macrophages infected with *Lysteria monocytogenes* are a source of IL-12, which subsequently directs Th1 development of naive $CD4^+$ T cells (10, 11), and that this effect of IL-12 seems to be partially mediated by NK cells (7, 12).

Concerning Th2 responses, even though the role of IL-4 in priming naive T cells for subsequent Th2 differentiation is well established (13, 14), the cellular source of IL-4 following encounter of the allergen or helmith parasites is unclear. It has been speculated that mast cells or basophils might be the initial source of IL-4, and that the interaction of helminths or allergens with these cells may induce IL-4 release (8, 9).

We investigated this possibility using bee venom phospholipase A_2 (PLA_2),[5] the main allergen involved in bee venom anaphylaxis (15–17). This Ag has previously been shown to elicit IgE and IgG Ab responses in mice and humans (15, 18), and its structural, functional, and immunologic characteristics have been studied extensively (19, 20). Since phospholipase A_2 enzymes have been shown to induce mediator release from mast cells (21), we reasoned that PLA_2 might trigger mast cells production of mediators such as IL-4 in an enzyme mediated mechanism. To test this hypothesis, we have generated a PLA_2 variant devoid of enzymatic activity by mutation of a catalytically conserved residue and have compared its ability to activate mast cells with its wild-type counterpart. We show that enzymatically active PLA_2, but not its catalytically inactive variant, induces nonimmunologic mediator release, including histamine and IL-4, from cells of rodent mast cell lineage. By comparing their immunogenicity in vivo, we further demonstrate that the catalytically inactive variant is unable to induce detectable IgE Ab synthesis under conditions in which its wild-type counterpart induces maximal IgE responses. The findings indicate that the enzymatic function of PLA_2 profoundly affects its immunologic behavior and suggest that mast cells might play a prominent role in the initiation of IgE Ab responses against this allergen.

[5] Abbreviations used in this paper: PLA_2, phospholipase A_2 from bee venom; CD, circular dichroism; 5-HT, 5-hydroxytryptamine; RBL, rat basophilic leukemia (cells); hIgE-V_{NP}, (4 hydroxy-3-nitrophenyl)acetyl-specific human IgE; HSA/NP, (4 hydroxy-3-nitrophenyl)acetyl coupled to human serum albumin; LDH, lactate-dehydrogenase; hFcϵR1α, human high affinity IgE receptor α-chain.

Materials and Methods

Reagents, enzymes, and general procedures

Chemicals of the highest available grade were purchased from Fluka (Buchs, Switzerland) or Sigma Chemical Co. (St. Louis, MO). DNA modifying enzymes were obtained from Boehringer Mannheim (Mannheim, Germany) and used as recommended. Plasmid DNA was prepared with anionic exchange columns (Qiagen kit, Qiagen Inc., Düsseldorf, Germany). Double-stranded DNA sequencing was performed according to the dideoxy chain-termination method (22) on an Applied Biosystems (Foster City, CA) 373A sequencer using the Taq DyeDeoxy Terminator cycle sequencing kit supplied by the same company. The type III/type IV and reverse primers (Qiagen) were used for sequencing.

Recombinant PLA_2 concentrations were determined according to the Bradford method, using a commercial kit (Bio-Rad, Richmond, CA) and commercial PLA_2 as a standard, or were calculated from the absorbance using an $\epsilon_{280}^{1\%}$ of 13.0 (23).

Recombinant antigens

Ags were expressed in *Escherichia coli* strain M15 bearing the pRep4 repressor plasmid (24), using the pDS56/RBSII, 6xHis expression plasmid (25) (kindly provided by Dr. D. Stüber, Hoffmann-La Roche, Basel, Switzerland). The expression of a synthetic gene encoding PLA_2 in this system and the isolation and characterization of recombinant wild-type Ag have been described in detail (26, 27).

The site-specific substitution of the catalytically essential and conserved histidine residue 34 with glutamine (H34Q) was introduced by PCR. A gene fragment carrying the H34Q mutation was amplified using the primer 5′-ACGCATGCTGTCGCACCCAGGACA-3′ together with the flanking T7 primer (Stratagene, La Jolla, CA) and the synthetic gene encoding the wild-type PLA_2 polypeptide in a pBluescript II SK vector as the target (26). PCR was performed in 100 μl of sample containing the appropriate buffer, 200 μM of each deoxynucleotide triphosphate, 0.5 μM of each 5′- and 3′-primer, 10 ng of target DNA, and 1 U of Vent DNA polymerase (New England Biolabs, Schwalbach/Taunus, Germany). The samples were subjected to two cycles of amplification consisting of denaturing for 1 min at 95°C, annealing for 1 min at 45°C, and extending for 1 min at 72°C, followed by 25 cycles under identical conditions except that the annealing was done at 50°C. The PCR-product was purified by agarose gel electrophoresis and recovered with GeneClean (Bio 101 Inc., La Jolla, CA). This mutant gene fragment was digested with *SphI* and *HindIII* and cloned into the same restriction sites of the wild-type PLA_2 expression construct (26). The presence of the mutation was confirmed by DNA sequencing, and constructs carrying the correct insert were selected for protein expression. The active site mutant PLA_2-H34Q was isolated, refolded, and purified as described for the wild-type counterpart (26, 27). Fresh resin and disposable plastic ware were used to exclude contamination of the mutant protein preparation with wild-type PLA_2.

Enzymatic assays

Enzymatic activities of the PLA_2 preparations were measured using the spectrophotometric assay with 2,3 bis-(hexanoylthio)propyl-1-phosphomethanol as substrate, following the previously described procedure (27) or using the pH-stat assay and 1,2 dimyristoyl-*sn*-glycero-3-phosphomethanol vesicles as a substrate (26).

Circular dichroism spectra

CD spectra were obtained using a J-720 spectropolarimeter (JASCO Instruments, Tokyo, Japan). The protein samples were dissolved in 10 mM sodium phosphate buffer, pH 7.0, at a concentration of 10 μM. Spectra

(average of 12–16 scans) were collected over a range of 180 to 300 nm using a 0.2-mm path length cuvette.

Phospholipid vesicle binding

The binding affinity of PLA_2 and PLA_2-H34Q to phospholipid vesicles was measured by fluorescence resonance energy transfer between tryptophan residues of the enzyme and the probe *N*-dansyl-hexadecylphosphoethanolamine present at 2 mol % in co-vesicles of the nonhydrolyzable phospholipids 1,2-ditetradecyl-*sn*-glycero-3-phosphomethanol (20%)/1,2-ditetradecyl-*sn*-glycero-3-phosphocholine (80%) (100 μM total phospholipid) in 10 mM Tris, 0.5 mM $CaCl_2$, pH 8.0 (28).

Antibody binding analysis

Relative binding affinities were determined by inhibition ELISA conducted as described (27). Briefly, purified recombinant Ag preparations were coated (0.1 μM diluted in PBS, pH 8.0; 100 ml/well) overnight at 4°C onto polystyrene microtiter plates (Maxisorb, Nunc, Denmark) followed by blocking of the remaining free sites with blocking buffer (PBS, pH 7.4, 1% casein hydrolysate (Oxoid Ltd., Basingstoke, U.K.), 5% (w/v) Tween 20). PLA_2-specific mAb at appropriate dilution was preincubated for 2 h at 37°C in a separate, untreated plate with serially diluted wild-type PLA_2 or PLA_2-H34Q. Thereafter this mixture was transferred to the Ag-coated plate and allowed to react for 2 h at 37°C. Bound Abs were detected with alkaline phosphatase-labeled secondary Ab followed by addition of 100 μl of substrate and evaluated on the kinetic microtiter plate reader (27).

Release of 5-hydroxytryptamine (5-HT) and hexosaminidase from rat basophilic leukemia (RBL) cells

RBL-2H3 cells transfected with the gene for the human Fcε receptor 1 α-chain (RBL-2H3 2/2/C) (29) were incubated in 24-well Costar (Cambridge, MA) plates ($2–4 \times 10^5$ cells/well) in a humidified environment of 5% CO_2 at 37°C for 24 h in a reaction volume of 0.4 ml with S-MEM (Life Technologies, Paisley, Scotland) containing 10% FCS, 5-HT (1 μCi/ml), 10^{-6} M dexamethasone in the presence or absence of serum from a bee venom-sensitive individual (diluted 1:10, estimated by ELISA to contain 400 ng/ml total IgE). Following this incubation period, cells were washed twice with 1 ml buffer A (120 mM NaCl, 5 mM KCl, 25 mM Pipes, 1 mM $CaCl_2$, 0.04 mM $MgCl_2$, 5.6 mM glucose, pH 7.4) and preincubated for a further period of 10 min at 37°C with the above buffer (0.5 ml). For the determination of 5-HT release, duplicate samples of cells were incubated with buffer A supplemented with 5′-(*N*-ethyl)carboxyamidoadenosine (100 μM) and PLA_2 or PLA_2-H34Q (0.01–100 μg/ml) at 37°C for 15 min. After cooling on ice, the supernatant was removed, and aliquots were removed for liquid scintillation counting. Release of hexosaminidase was determined as described (30). Percent release was calculated by the method of Siraganian (31). All data are normalized with regard to the value obtained for triggering with 1 μg/ml (4 hydroxy-3-nitrophenyl)acetyl-specific human IgE (hIgE-V_{NP}) and 100 ng/ml (4 hydroxy-3-nitrophenyl)acetyl coupled to human serum albumin (HSA/NP) (29).

IL-4 production by RBL-2H3 (2/2/C) cells

RBL-2H3 (2/2/C) cells (29) were incubated in 6-cm petri dishes ($\sim 2.5 \times 10^6$ cells/dish) in a humidified environment of 5% CO_2 at 37°C for 24 h in a total reaction volume of 2.5 ml with S-MEM, FCS (10% v/v). Following incubation, cells were washed twice with 2.5 ml of buffer A (see above) and preincubated for a further period of 10 min at 37°C with the same buffer (2.5 ml) before challenge with buffer A supplemented with 50 μg/ml PLA_2 or PLA_2-H34Q. After the indicated incubation period, plates were cooled on ice. The cell supernatant was removed, and the corresponding cell pellets were solubilized in 2.5 ml buffer A containing 0.1% Triton X-100. Aliquots of the cell pellet and supernatant fractions (1.5 ml) were precipitated with 4.5 ml of acetone (−20°C for 15 min), and the precipitate was collected by centrifugation (14,000 × *g* for 10 min). The pellets were dissolved in 100 μl of SDS sample buffer and proteins were separated by 17% SDS-PAGE (32) and blotted onto nitrocellulose (33). IL-4 immunoreactive protein was detected with an antiserum raised in rabbits against the rat IL-4-derived peptide DSPLREI INTLNQV. A FAST search (34) of the database (SWISSPROT version 32.0) using this sequence revealed no significant sequence similarity with other known proteins. Rabbits were immunized s.c. with 0.5 mg/ml of peptide emulsified in CFA, followed by two injections in IFA (s.c, 0.5 mg/ml) at 15-day intervals. Four days after a final boost with peptide in PBS (i.v., 0.5 mg/ml), serum was collected and Ab titer was determined by ELISA. This antiserum recognizes at least three ~18- to 21-kDa proteins secreted from CHO.K1 cells transfected with the gene encoding rat IL-4 (gift from Drs. A. N. Barclay and M. Puklavec, Medical Research Council (MRC), Oxford, U.K.) (35) on Western blots. An antiserum raised in rabbits against recombinant rat IL-4 (generous gift from Dr. M. Puklavec, MRC) recognized the same bands on immunoblots.

IL-4-associated biologic activity was measured by induction of MHC class II/Ia expression on rat B splenocytes. To obtain B splenocytes, rat spleen cells were washed twice in serum-free RPMI 1640 medium, followed by flash lysis with 5 mM of NH_4Cl (2 ml) at room temperature for 1 min. After addition of 20 ml of RPMI, the cell suspension was centrifuged for 5 min at 1000 rpm, cells were resuspended in 600 μl of PBS/0.5% BSA, and 100 μl of the following Abs to T cell markers were added: W3/25, W3/13, OX8, and OX-35 (all obtained from Serotec, Oxford, U.K.). The cells were incubated for 1 h at 4°C with gentle shaking, washed twice with 20 ml of RPMI and resuspended in 500 μl of PBS/0.5% BSA. Dynabeads M450 coated with sheep anti-mouse IgG (Dynal 110.01, 500 μl) were added, and cells were incubated for 1 h at 4°C with gentle shaking. Coated T cells were depleted using a magnetic particle concentrator, and the supernatant containing splenic B cells was collected. Following washing with RPMI, B cells were suspended in serum-free medium (Macrophage-SFM, Life Technologies) at a cell density of 7×10^5 cells/ml. Aliquots (300 μl/well) of the cell suspension were plated out in a 24-well Costar plate, and 100 μl of the supernatants released from RBL-2H3 2/2/C cells in response to incubation with PLA_2 and PLA_2-H34Q were added to each well. The spleen cells were incubated at 37°C in a humid atmosphere with 5% CO_2. After 24 h, cells were washed twice with serum-free RPMI and incubated for 1 h at 4°C with FITC-labeled Ab to rat class II/Ia molecules (OX6, Serotec U.K., 1:25 dilution in PBS/5% BSA). Before analysis by flow cytometry, cells were washed twice with PBS/1% BSA and resuspended in 500 μl PBS/1% paraformaldehyde. An unrelated FITC-labeled Ab (anti-human IgE) was used to set the threshold for MHC class II positive staining.

Immunization of mice

CBA/J mice (6–8 wk of age) were purchased from Bommice (Bomholtgard, Ry, Denmark) and were immunized by biweekly i.p. injections of low dose (LD: 0.1 μg/injection) or high dose (HD: 10 μg/injection) of PLA_2 or PLA_2-H34Q in 2 mg of $Al(OH)_3$. Sera were collected at day 35 after the first immunization and assayed for PLA_2-specific IgE and IgG Abs by ELISA. Assays were performed as described (18), except that standards for IgG (anti-PC-12–3) and IgE (anti-PC-56–1) (36) were included. Titers were determined by twofold serial dilutions of the sera starting with 1:50, and are defined as the serum dilution giving a signal equivalent to 0.4 ng/ml of IgE and IgG standard, respectively.

Results

IL-4 is known to play a central role in the induction and control of IgE Ab responses. However, the cellular origin of IL-4 as well as the mechanisms by which certain Ags stimulate the synthesis of this cytokine at a time when specific lymphocyte responses are not yet established are still unclear. Besides T lymphocytes, mast cells and basophils have been shown to produce considerable amounts of IL-4 following stimulation (37–39). The present study investigates the possibility that allergens might directly activate cells of mast cell/basophil lineage and induce IL-4 production by a nonimmunologic mechanism.

We used PLA_2, the main allergen of bee venom, to explore this possibility. Since endogenous phospholipase A_2 enzymes have been shown to cause mast cell activation

(21, 40), we hypothesized that PLA_2 might activate mast cells by virtue of its enzymatic activity. We therefore generated PLA_2-H34Q, a PLA_2 variant lacking enzymatic function, by substituting the catalytically essential and evolutionary conserved histidine residue 34 (19, 20) with glutamine, and compared its mast cell secretagogue activity to its enzymatically active wild-type counterpart.

Both PLA_2 and PLA_2-H34Q were isolated from the same *E. coli* high level expression system (26). As predicted by the proposed enzymatic mechanism (19), PLA_2-H34Q exhibited no detectable enzymatic activity ($<10^{-4}$ of wild-type-specific activity). However, PLA_2 and PLA_2-H34Q had comparable secondary structure as assessed by CD spectroscopy. Mutant and wild-type PLA_2 lack free thiols when analyzed with Ellmann's reagent and mass spectrometry (26), indicating that all five disulfide bonds have been formed. Fluorescence energy transfer experiments show that wild-type and mutant PLA_2 display tight and saturable binding to phospholipid vesicles (Fig. 1*A*). While this type of analysis will not yield an accurate dissociation constant because the affinity is so high, it is apparent that both PLA_2 and PLA_2-H34Q bind to phospholipid vesicles with nanomolar affinity, which is a hallmark of 14-kDa phospholipases A_2 (41). It is also apparent that the efficiency of energy transfer is comparable for both molecules, suggesting that position and orientation of the tryptophan residue involved in energy transfer is not significantly altered. Immunologic analysis shows that mutant and wild-type Ag exhibit the same affinity to a conformation-specific human mAb directed against the K25 epitope (27) (Fig. 1*B*). In addition, PLA_2 and PLA_2-H34Q provoke comparable type I skin reactions in bee venom allergic individuals (42). Thus, PLA_2-H34Q folds into a native-like conformation, and the lack of enzymatic function is not due to a disruption of the correct three-dimensional structure.

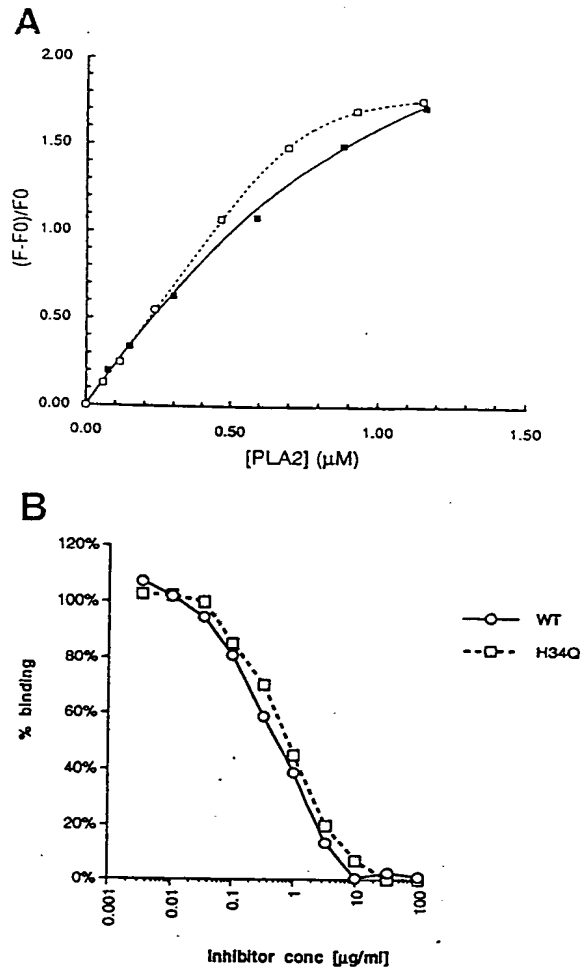

FIGURE 1. *A*, Binding of PLA2 and PLA2-H34Q to phospholipid vesicles. Binding affinity was estimated from the relative fluorescence emission enhancement due to energy transfer from tryptophan side-chains on PLA2 (closed symbols) or PLA2-H34Q (open symbols) to the dansyl fluorophore present at 2 mol % in nonhydrolyzable phospholipid vesicles as described in *Materials and Methods*. *B*, Binding of PLA2 and PLA2-H34Q to the conformation-dependent human mAb BVA1 specific for the K25 epitope (27). Relative binding affinities were determined by inhibition ELISA, in which serially diluted PLA2 or PLA2-H34Q was preincubated with BVA1 followed by transfer of the mixture to a wild-type Ag-coated plate. Following incubation, Ab binding was measured as described in *Materials and Methods*. The extent of binding is expressed as percent of reactivity in the absence of inhibitor, after subtracting background for each point.

Degranulation of RBL cells

We first tested the ability of PLA_2 and PLA_2-H34Q to degranulate rat basophilic leukemia cells transfected with the gene encoding the α-chain of the human high affinity IgE receptor complex (hFcϵR1α) (RBL-2H3 2/2/C clone) (29) in the presence or absence of sensitization with serum from an individual allergic to bee venom. RBL cells represent an accepted model system for the study of mucosal mast cell function (43). Following sensitization, both PLA_2 and PLA_2-H34Q induce mediator secretion, as monitored by the release of [^{3}H]5-hydroxytryptamine (5-HT), from transfected cells with comparable efficiency, as demonstrated by the bell-shaped dose-response curve observed for both Ags (Fig. 2). This dose-response curve is characteristic of mast cell stimulation induced by receptor-bound IgE cross-linking. The comparable potency of PLA_2 and PLA_2-H34Q shows that both Ags are equally recognized by IgE Abs and provides additional confirmation that PLA_2-H34Q displays no significant change in the tertiary conformation.

In contrast, when hFcϵR1α transfectants are incubated with the same concentration of Ag in the absence of sensitizing serum, active PLA_2 causes degranulation (Fig. 2), and mediator secretion increases with Ag dose, whereas

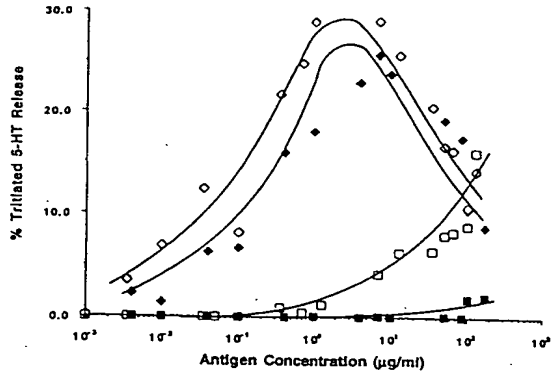

FIGURE 2. 5-HT release from RBL-2H3 2/2/C cells incubated with PLA2 and PLA2-H34Q. RBL-2H3 cells transfected with the gene for hFcεR1α (29) with (diamonds) or without (squares) sensitization with allergic human serum were challenged with varying amounts of PLA2 (open symbols) or PLA2-H34Q (closed symbols). 5-HT release was measured after a 15-min incubation at 37°C. Percent release was calculated by the method of Siraganian (31). Each symbol represents the mean of two or three tests carried out in duplicate. All data are normalized with regard to the value obtained for triggering with 1 μg/ml of hIgE-V_{NP} and 100 ng/ml of HSA/NP (29).

the inactive variant PLA_2-H34Q induces only marginal release at the highest Ag concentration. Release patterns similar to those observed for 5-HT were obtained for hexoseaminidase (data not shown). Thus, the enzymatic activity of PLA_2 seems to promote IgE-independent mast cell activation.

Induction of IL-4 synthesis in RBL cells

Previous reports demonstrated de novo protein synthesis and secretion of cytokines, including IL-4, by mast cells in response to FcεR1 cross-linking (37, 38). Since the enzymatic activity of PLA_2 appears to promote IgE independent mast cell activation, we monitored, over a period of 6 h, the protein content in pellets and supernatants of RBL cells that had not been sensitized with IgE, before and after addition of PLA_2 and PLA_2-H34Q to the medium bathing the transfected cell line. During this period, the protein concentration in the supernatants of cells exposed to PLA_2 rises from ~30 μg/ml to ~390 μg/ml. The corresponding increase in unstimulated cells or those exposed to PLA_2-H34Q is less than 50 μg/ml.

The presence of IL-4 in the cell pellet and supernatant fractions of these samples was demonstrated by Western blot analysis using an antiserum raised in rabbits against a rat IL-4-derived peptide or a polyclonal rabbit anti-IL-4 Ab (generous gift of Dr. M. Puklavec, MRC Oxford). The immunoblot (Fig. 3) shows that PLA_2 stimulates a time-dependent expression and release of IL-4 from RBL 2H3 2/2/C cells. Several polypeptide bands of ~18 to 22 kDa

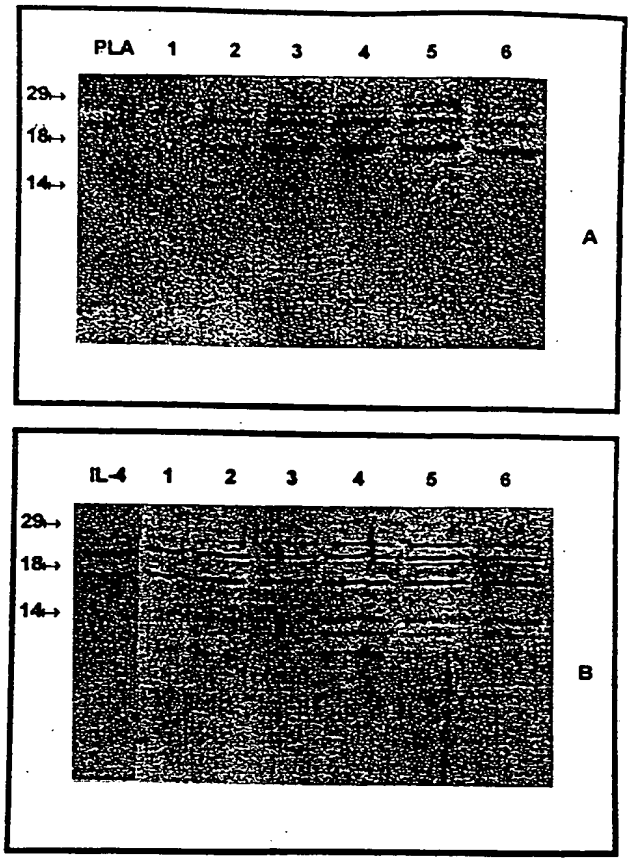

FIGURE 3. Kinetics of IL-4 induction in RBL-2H3 (2/2/C) (29) cell pellets and release following exposure to PLA2. RBL-2H3 (2/2/C) cells (29) were challenged with 50 μg/ml of PLA2 or PLA2-H34Q in the absence of sensitizing IgE. After the indicated incubation period, proteins present in culture supernatants or cell pellets were resolved by SDS-PAGE and analyzed by immunoblotting with Ab to IL-4-derived peptide. Anti-IL-4 Ab was detected by horseradish peroxidase-labeled anti-rabbit Ab. The *first lane* in *panel A* represents the control for PLA2 in buffer A employed for cell challenge, and in *panel B* the *first lane* corresponds to the supernatant of CHO.K1 cells transfected with the gene encoding rat IL-4 (35). *Lanes 1–6* represent cell supernatants (*A*) and pellets (*B*) before and 15, 60, 120, 240, and 360 min after challenge with PLA2. No IL-4 immunoreactivity was detected in cell supernatants, and no increase of the basal cellular levels of IL-4 was observed following challenge with PLA2-H34Q (not shown). Comparable results were obtained using an Ab raised against recombinant rat IL-4 (generous gift from Dr. M. Puklavec, MRC).

are detected in cell pellets; they probably represent the different glycosylated forms of IL-4 reported by others (35) and pro-IL-4. Within 15 min after addition of PLA_2, the transfectants secrete detectable quantities of IL-4 into the cellfree fraction. Additional bands of higher mobility appear within an hour in the cell pellet, but not in the supernatant fraction, of PLA_2-stimulated cells. These are

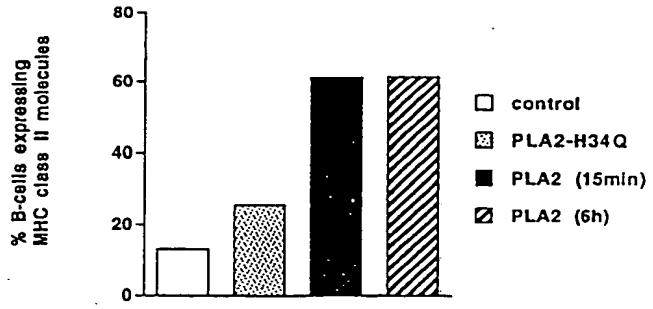

FIGURE 4. Up-regulation of MHC class II expression on rat B splenocytes by RBL-2H3 supernatants. Splenic B cells were isolated through immunomagnetic depletion of splenic T cells, as described in *Materials and Methods*. After isolation, B splenocytes were incubated with supernatants of RBL cells that have been treated with buffer alone (control) or challenged with 50 μg/ml of PLA2-H34Q (15 min) or PLA2 for 15 min and 6 h, respectively. Following incubation, B splenocytes were stained with FITC-labeled Ab to rat class II molecules and analyzed by flow cytometry. The plot shows the % MHC class II-positive B cells following treatment with RBL supernatants, as described in *Materials and Methods*.

most likely incomplete translation products and point to a stimulation of de novo protein synthesis, which peaks between 2 and 4 h. Cells incubated with PLA_2-H34Q under identical conditions do not respond with mediator secretion, and no increase in cellular IL-4 is observed during the 6-h period (data not shown). These observations indicate that PLA_2 fuels rapid secretion of IL-4 and transient increase in IL-4 synthesis, a finding that had been made earlier when mast cells and basophils responded to an immunologic stimulus (37–39).

To obtain independent evidence for IL-4 production by RBL 2H3 cells following nonimmunologic stimulation by PLA_2, we measured MHC class II expression on primary rat B splenocytes in response to supernatants of RBL 2H3 cells. Up-regulation of class II Ag expression on B splenocytes is a characteristic action of IL-4 (35). Figure 4 shows that only mast cells exposed to PLA_2 secreted component(s) that enhanced expression of MHC class II/Ia Ags, whereas class II/Ia expression induced by supernatants of RBL 2H3 cells challenged with PLA_2-H34Q increased only marginally. Moreover, a polyclonal rabbit anti-IL-4 Ab (generous gift of Dr. M. Puklavec, MRC) blocked MHC class II up-regulation (data not shown), providing compelling evidence that rat mast cells secrete IL-4 in response to PLA_2 in the absence of IgE sensitization.

Induction of IgE Ab response in vivo

To test whether this enzyme-induced mediator secretion by PLA_2 might be of significance for Ag-specific immune responses in vivo, we compared isotype-specific Ab responses elicited by PLA_2 and PLA_2-H34Q following immunization of mice. Previous investigations employing purified natural PLA_2 have shown that CBA/J mice are high responders with respect to both IgG and IgE Ab production. These studies further established that the prevalence of the isotype is critically dependent on the Ag dose (18): repeated injections of low doses of Ag (0.1 μg/injection) induced high titers of PLA_2-specific IgE, whereas immunizations with high Ag doses (10 μg/injection) elicited high levels of Ag-specific IgG. We followed the immunization schemes established by this model to assess the influence of the enzymatic activity on isotype selection in the Ab response against PLA_2. The results of a typical experiment in which identical sets of high Ag dose and low dose immunization procedures were conducted in CBA/J mice using PLA_2 and PLA_2-H34Q as Ags are shown in Figure 5. The outcome demonstrates that repeated injections with low doses of wild-type Ag induces high levels of PLA_2-specific IgE, while immunization with PLA_2-H34Q under identical conditions yields no detectable PLA_2-specific IgE synthesis. In the latter case, PLA_2-specific IgG levels were significantly lower compared with those elicited by wild-type Ag, but detectable IgG1 and IgG_{2a} responses were found in every animal.

In contrast, when high doses of Ag were used for immunization, the isotype pattern and specific Ab levels were found to be similar for both PLA_2 and PLA_2-H34Q. These results indicate that PLA_2 and PLA_2-H34Q are similarly immunogenic at high dose; at low dose, however, enzymatically active PLA_2 induces high titer IgE Ab responses under conditions in which PLA_2-H34Q is only weakly immunogenic.

To assess whether the differences observed reflect merely a shift in the dose-response profile between PLA_2 and PLA_2-H34Q, mice were immunized with intermediate doses of mutant Ag. Although PLA_2-H34Q exhibits a gradual increase in immunogenicity, as shown by the dose-dependent rise in specific IgE and IgG levels, a high titer IgE response comparable to the wild-type Ag is not induced by any dose of PLA_2-H34Q. Specific IgE levels reached a maximum in mice immunized with 1 μg/injection, and titers were equal to those observed in mice immunized with 10 μg/injection of Ag. Thus, the high titer IgE Ab response of PLA_2 appears to be intrinsically associated with its enzymatic activity and does not occur with any dose of its enzymatically inactive counterpart.

Discussion

A central issue in the development of IgE-mediated allergies is the characteristics of an allergen that promotes IL-4 synthesis at a stage where specific lymphocyte responses are not yet established and thus drives Ab responses towards a predominant IgE isotype. Regarding the cellular source of IL-4 at the onset of immunity, a recently identified $CD4^+NK1.1^+$ rare T cell population, which promptly produces IL-4 following challenge with anti-CD3 or superantigen, has been proposed to play a role in

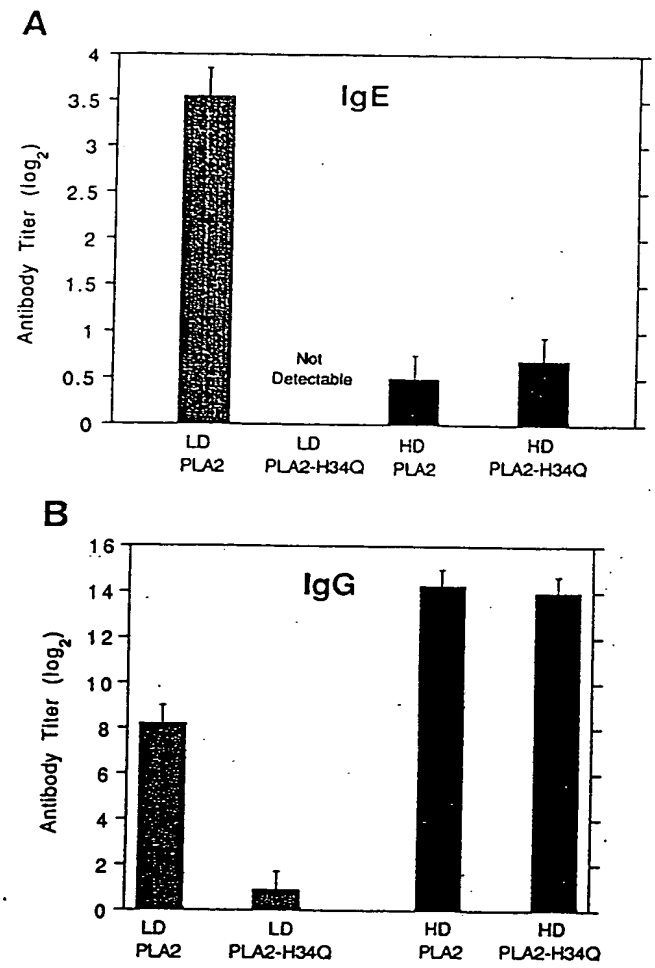

FIGURE 5. Isotype pattern of PLA2-specific Abs induced by PLA2 and PLA2-H34Q. CBA/J mice were immunized with low dose (LD, 0.1 μg/injection) or high dose (HD , 10 μg/injection) of Ag. Sera were collected at day 35 after the first immunization and assayed for PLA2-specific IgE (*A*) and IgG (*B*) Abs by ELISA. At this time the isotype pattern is established and Ab titers do not change thereafter. Ab titers were determined by ELISA and were normalized using IgE and IgG reference standards as described in *Materials and Methods*. Each bar represents the arithmetic mean (+SE) of a group of four animals, and data are expressed as log_2 of Ab titers. Comparable isotype patterns were observed in three sets of immunizations.

IL-4-dominated immune responses; it has been speculated that allergens or nematodes might have associated superantigens that enable them to induce IL-4 production during the priming of antigen-specific T cells (44). Alternatively, the source of IL-4 at the onset of immunity might be provided by mast cells or basophils, and it has been speculated that the interaction of helminths or allergens with these cells may induce IL-4 release (8, 9). The difficulty with the later possibility is that the only known physiologic stimulus inducing IL-4 production by these cells is cross-linking of FcεR1 (37–39), and this would normally occur only after specific IgE Ab responses are already established.

In the present study we investigated the possibility that an Ab-independent interaction of an allergen such as PLA_2 with mast cells or basophils might also stimulate IL-4 production. In the light of the suggested role of endogenous phospholipase A_2 enzymes in mast cell activation (21, 40), we hypothesized a direct enzymatic action mode of the PLA_2 allergen in inducing mast cell activation. Thus, we measured mast cell stimulation by PLA_2 and PLA_2-H34Q, a mutant that is structurally and functionally indistinguishable from wild-type Ag except that it lacks enzymatic activity. We have shown that in rodent mast cells PLA_2 triggers IgE-independent mediators release (Fig. 2) and induces de novo synthesis and secretion of IL-4 (Figs. 3 and 4), demonstrating that allergens can stimulate IL-4 production by cells of mast cell lineage by a nonimmunologic mechanism. The finding that these activities cannot be observed with the enzymatically inactive variant further suggests that the stimulatory effect is due to the enzymatic function of PLA_2.

The simplest interpretation of all these findings would be that PLA_2 activates mast cells in a direct enzyme-mediated process by hydrolyzing membrane-bound phospholipids, and that the products of phospholipid hydrolysis might trigger mast cell degranulation. In support of this view, endogenous type II phospholipase A_2 has been associated with mast cell degranulation (21, 40). Thus the PLA_2 allergen, by virtue of its lipolytic activity, may simply mimic the metabolic signals normally generated by the cellular phospholipase A_2 and bypass the cellular control of mast cell activation.

It might be argued that mediator release from RBL cells could result from a loss of cell integrity due to membrane phospholipid degradation by PLA_2. We have addressed this issue by measuring release of lactate-dehydrogenase (LDH), a cellular enzyme released upon cell disruption into culture supernatants following incubation with 50 μg/ml of PLA_2. We found that, during a 2-h incubation period under conditions in which mediator release and IL-4 secretion occurs, <8% of the LDH was released from RBL cells. In contrast to that, incubation of RBL cells under identical conditions with 50 μg/ml of bee venom resulted in 100% LDH release and in a visible loss of cell integrity. Therefore, we believe that the cytotoxic effects reported for (commercially available) PLA_2 by some investigators in the past are most likely due to contamination with other bee venom components such as mellitin, a known cytolytic peptide which is abundant in bee venom.

Another hypothesis would be that the PLA_2 allergen activates mast cells by binding to a putative receptor. Recent studies have identified a high affinity receptor for type I phospholipase A_2 in various tissues (45, 46), and a brain receptor for potent neurotoxins has been shown to bind several phospholipase A_2 enzymes from various species

with high affinity (47, 48). Interestingly, the bee venom enzyme has also been reported to bind to a neurotoxin receptor in rabbit skeletal muscle with nanomolar affinity (49). Since the only conserved structural feature of these diverse phospholipase A_2 enzymes lies in the catalytic site, it is conceivable that PLA_2 might bind to these receptors via its active site. It is presently unknown, however, whether these receptors are also present on mast cells.

The observed link between enzymatic activity and IgE-independent mast cell activation by PLA_2 implies that only catalytically active Ag should efficiently initiate IgE Ab synthesis in vivo, a prediction we confirm using the experimental murine system.

In Figure 5 we show a preferential induction of IgE Abs as a result of repeated low dose immunization with enzymatically active, but not inactive, PLA_2. It should be noted that under low dose immunization conditions, PLA_2-H34Q is considerably less immunogenic than its wild-type counterpart, whereas no difference in IgG or IgE responses is apparent when animals are immunized with high doses. Thus, the enzymatic activity of PLA_2 is not only associated with predominant IgE responses but also leads to an increased immunogenicity at low dose. It could be argued that the difference in immunogenicity might be due to the disruption of a dominant T helper cell epitope by the H34Q mutation. When the proliferative response of lymph node cells from mice immunized with 0.1 μg/injection of PLA_2 was measured in vitro after stimulation with 10 μg/ml PLA_2, PLA_2-H34Q, or control buffer, wild-type and mutant Ag incorporated 32,933 $\pm$ 1,616 and 32,890 $\pm$ 1,821 cpm, respectively, compared with 543 $\pm$ 312 cpm in the buffer control.

Thus, we find no evidence of an immunodominant T helper cell epitope spanning the region of H34, but since the T cell epitopes on PLA_2 in this or any other mouse strain are at present unknown, we cannot rule out the possibility that an altered T helper epitope can contribute to the different immunogenicities observed in vivo. In the light of the demonstration of enzyme-mediated activation mast cells, however, we favor a direct enzymatic action mechanism in which PLA_2 induces IL-4 synthesis by mast cells during the priming of specific lymphocyte responses, which finally results in a predominant IgE Ab response.

The present investigation has focused on the biologic action of the Ag on rodent mast cells and IL-4 secretion; the release of additional mediators from these and possibly other cell types in response to Ag will be considered in further investigations. It is interesting to note in this context that many airborne and food-associated sources of allergens comprise lectins and enzymes such as phospholipases and proteases (2, 50, 51). The latter are also released by invading parasites, bacteria, and viruses. We and others have shown that proteases, including those of house dust mite, fungal, bacterial, or schistosomal origin, can degranulate mast cells by a nonimmunologic mechanism (2, 50, 51). This suggests that a common mechanism may determine the isotype response to allergens or parasite Ag: interesting to note in this context that dose-dependent is responses comparable to those elicited in response to PLA_2 were observed when the infection by the nem *Nippostrongylus brasiliensis* was studied in rats (52). firmation of this hypothesis should have important c quences for the design of vaccination schedules to pr IgE-mediated allergies and parasitic infestations.

Acknowledgments

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Assessment of the Molecular Basis of the Proallergenic Effects of Cigarette Smoke

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Assessment of the Molecular Basis of the Proallergenic Effects of Cigarette Smoke

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Epidemiological studies indicate a link between smoking and increased risk of immunoglobulin E-mediated allergies and asthma. The molecular basis underlying cigarette smoke related respiratory disorders are ill defined, but it is known that mast cells in the mucosal lining of the airways are an important reservoir of proinflammatory mediators, which play a pivotal role in the development of these diseases. The establishment of a novel cell exposure unit facilitated a study of mast cell responses to pollutants in mainstream cigarette smoke at the air/cell interface. Our study shows that cigarette smoke, but not filtered clean air, induces the release of mediators of type I hypersensitivity responses and stimulates the synthesis of proinflammatory cytokines, including interleukin (IL)-4, 5, 10, and 13 and tumor necrosis factor (TNF)-α, in cells of mast cell lineage. These results explain how exposure to pollutants present in cigarette smoke can induce the pathophysiological responses associated with allergy, IgE-mediated and IgE-independent asthma since IL-4 and IL-13 induce class switching to IgE, and IL-13 has recently been identified as the key mediator of IgE-independent asthma.

Introduction

Increased cancer risk as a result of long term exposure to cigarette smoke is well established. In addition, it has been extensively documented that cigarette smoking is responsible for initiating and/or exacerbating a variety of respiratory disorders, including chronic obstructive pulmonary disease, emphysema, and asthma (*1–5*). Active and passive smoking also increases the risk of development of occupational allergies, and parental smoking is linked to increased airway hypersensitivity and sensitization to common aero-allergens in children (*5–8*). The molecular mechanisms associated with cigarette smoke related type I hypersensitivity responses are not understood. However the r le of both interleukin (IL)-4 and IL-13 in the induction of antibodies of the immunoglobulin (Ig) E isotype is well established. In addition, recent evidence has been obtained which points to a key role of IL-13 in causing the symptoms of allergic asthma, which is independent of IgE and IL-4 (*9, 10*).

Because mast cells are extensively distributed in the mucosal lining of airways and an important source of potent mediators of airway inflammation (*11*), we monitored initially histamine, β-hexosaminidase (β-Hex), and IL-4 secretion in response to environmental tobacco smoke (ETS). Our preliminary investigations demonstrated histamine release from human lung mast cells (HLMC) and β-Hex secretion from rat basophilic leukemia cells (RBL) (an established model system for the study of mucosal mast cell function) exposed to cigarette smoke containing air. No degranulation was observed from cells maintained in a smoke free room, under otherwise identical conditions (D. C. Machado, Ph.D. Thesis, University of Sheffield 1994). The similarity of responses of HLMC and the RBL cell line to pollutants in cigarette smoke (and other environmental allergens reviewed in refs *12–14*) justify the use of the RBL J41 cell line (*14*) as a model system of mast cell responses to investigate the effect of cigarette smoke under defined exposure conditions and obviates the need to prepare primary HLMC (*12, 13*). Most importantly, the novel assay system employed in the present study facilitates direct exposure of cells maintained at the air/liquid interface to freshly generated mainstream cigarette smoke in the absence of an intervening layer of tissue culture medium (*15*). The experimental conditions, therefore, closely resemble cell exposure to the environment at mucosal surfaces.

Experimental Section

Cell Maintenance and Monitoring of Cellular Responses to Ambient Air. The preparation of dissociated cell suspensions containing HLMC and the culture conditions for the RBL cell line have been published (*12–14*). Release of histamine, 5-hydroxytryptamine (5-HT), or β-hexoseaminidase (β-Hex) and IL-4 release as an index of degranulation, and of lactate dehydrogenase (LDH), as an indicator of cytotoxicity, were determined using standard assays (*12, 13*). Secretory responses of target cells maintained in 24 well tissue culture dishes (Costar) covered with either 200 or 400 μL assay buffer (*12, 13*) were assessed following 4 h exposure to either cigarette smoke free ambient atmospheric air or to ETS containing enclosures. To study the effect of ambient air or cigarette smoke on mediator release, test and control cells were maintained in separate rooms (ambient temperature 22–24 °C, humidity ~85–90%, enclosed air space ~32 m³). Volunteers smoking a commercially available cigarette brand at regular intervals (1 cigarette/8 min) generated cigarette smoke, which was blown over the cells at a distance ~75 cm. The experiment began when cells were placed in the room, and the cover of the tissue culture plate was removed.

In addition, we monitored exposure to air containing cigarette smoke in a public bar (Bar 1, Sheffield University, ~300–350 people present, ~50–60% smokers, ambient temperature 24 °C, humidity ~85–90%, air space ~1300 m³).

Exp sure Syst m. The design of the exposure chamber and air phase dilution systems, including smoke generation and composition, have been described in detail elsewhere (*15*). Briefly, the cell exposure chamber allows direct exposure of cells cultured on collagen treated "Transwells" (Costar) to freshly generated and subsequently diluted mainstream cigarette smoke. Cigarette smoke exposure conditions fol-

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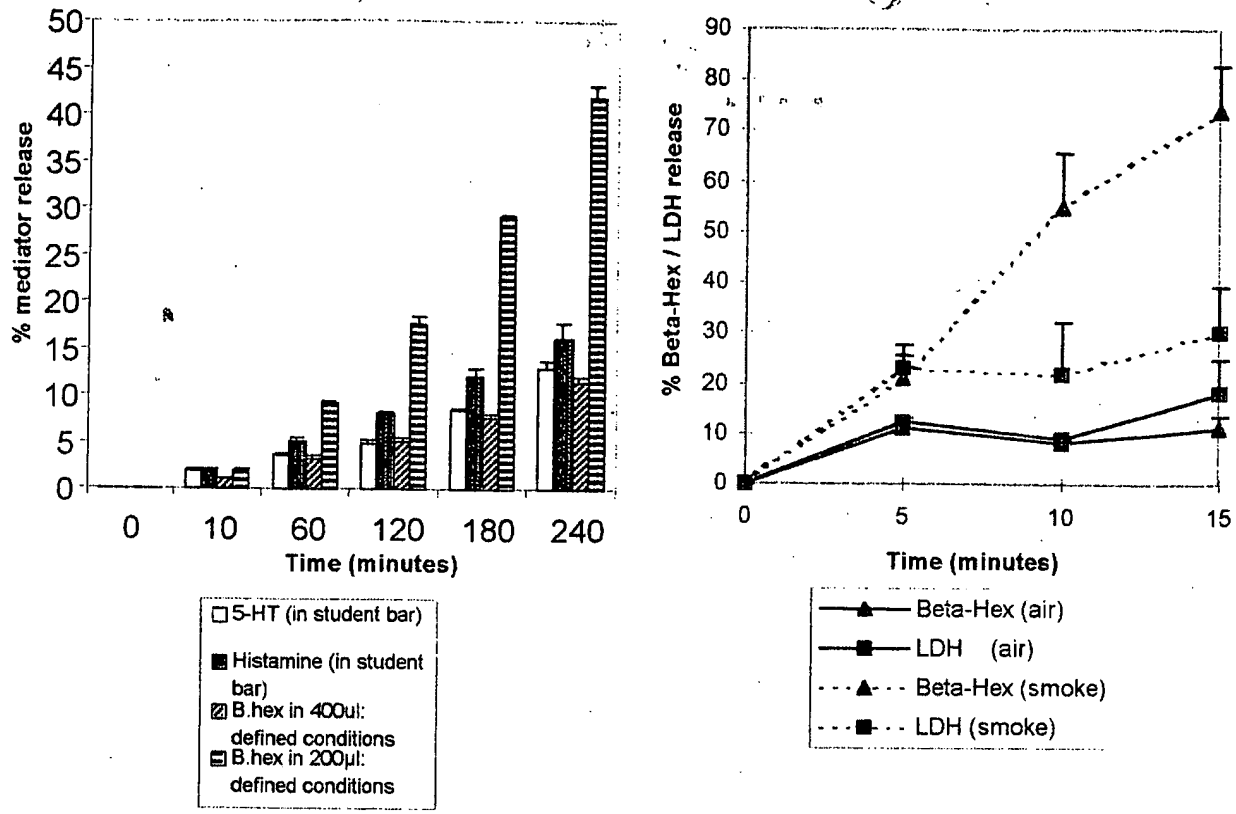

FIGURE 1. Comparison of mediator release from human lung tissue enriched in HLMC and RBL-J41 cells. a: Histamine release [■] from HLMC and 5-HT [□]/ β-Hex release [vertical lines, slanted lines] from RBL-J41 cells, plated in 24 well tissue culture dishes (Costar) and covered with either 200 or 400 µL buffer, was monitored after removal of the cover, during a 4 h exposure period to ETS generated by volunteers smoking a commercially available cigarette brand at regular intervals (32 cigarettes/4 h) in a secluded, unventilated room (ambient temperature ~24 °C, humidity ~85%, enclosed air space ~32 m³). Cells covered with 400 µL buffer were also exposed to ETS in a public bar (Bar 1, Sheffield University, ~300–350 people present, ~50–60% smokers, ambient temperature ~24 °C, humidity ~90%, enclosed air space ~1300 m³). Basal histamine/5-HT/β-Hex release (*12, 13*) from control cells kept in clean air was <4/2.5/1.2% during the period under investigation and subtracted from test values. Each point represents the mean (± SEM) of data from two and three independent determinations, respectively, carried out in duplicate for HLMC and RBL-J41 cells, respectively. ($P = 0.41$ for 5-HT values in bar environment verses β-Hex values in defined environment). b: β-Hex and LDH release from RBL-J41 cells, maintained at an air/liquid interface, were determined at 5 min intervals over a 15 min exposure period at 37 °C to Smoke or Air. Each point represents the mean (± SEM) of values from four independent determinations, each performed in duplicate. Paired T tests for a one tailed hypothesis at 95% confidence intervals show that LDH release is not significantly greater than β-Hex release when cells are exposed to Air or Smoke ($P > 0.05$); LDH release becomes significantly higher in Smoke exposed compared to Air exposed cells ($P < 0.05$). β-Hex release is significantly greater than LDH release when cells are exposed to smoke for >10 min ($P < 0.05$); β-Hex release increases significantly when cells are exposed to Smoke for >10 min compared to cells exposed to Air ($P < 0.05$).

lowed standard guidelines (ISO 3308; 3rd ed. 1991). The exposure equipment consists of a smoke generator, and the real time concentration of smoke particulate matter is monitored using an online aerosol photometer. The relationship between mass concentration of particulate matter in main stream smoke and the amounts of particulate material deposited on the "Transwell" membrane is well established and has been published (see Figure 3, ref *15*). RBL cells are cultured on a porous collagen substratum at the air/liquid interface and are subjected over a period of up to 15 min (equivalent to the duration of inhaling one cigarette) to periodic exposure to a mainstream smoke containing air phase. The tissue culture unit allows variation in the exposure protocol with regard to smoke concentration and duration of exposure. During smoke exposure, only the lower surface of each Transwell is supplied with tissue culture medium, while cells at the upper surface can be exposed directly to air or freshly diluted mainstream cigarette smoke in the absence of an intervening layer of tissue culture medium (*15*).

Exposure at the Cell/Air Interface. RBL-J41 (*15*) cells were plated onto collagen inserts of six well plates, "Transwells" (Costar) at 2.5×10^6 cells/well, 18–24 h before transfer of Transwells with adhering cells into the smoke exposure chamber. Cells were not sensitized with IgE. Exposure conditions were optimized to obtain high levels of mediator secretion, while minimizing cytotoxic effects. The smoke dilution setting of the apparatus, which is variable between 1:8 and 1:200, was set at 1:100 dilution with a cigarette smoke rate of 1 inhalation per min and exposure duration to mainstream smoke containing air of 16 s per min. The physical characterization of smoke under these conditions has been published (*15*). Cultured cells were exposed for a total of 0, 5, 10, or 15 min to periodic cigarette smoke (Smoke) or clean, filtered air (Air). After the specified exposure period, mediators secreted by exposed cells were collected immediately from the underlying tissue culture medium. Aliquots (50 µL) were assessed for LDH release and β-Hex activity (500 µL).

Statistics. Levels of mediator release were calculated as mean ± SEM. Paired t tests are for a one tailed hypothesis at a 95% confidence interval, where a value of $P < 0.05$ is considered significant.

cDNA Cloning and Gene Sequencing. Following exposure to Air or Smoke, RBL-J41 cells attached to collagen coated Transwells were submerged into growth medium (Dulbecco's Minimal Essential Medium, DMEM) and incubated for a further 1 h period at 37 °C in a standard tissue culture

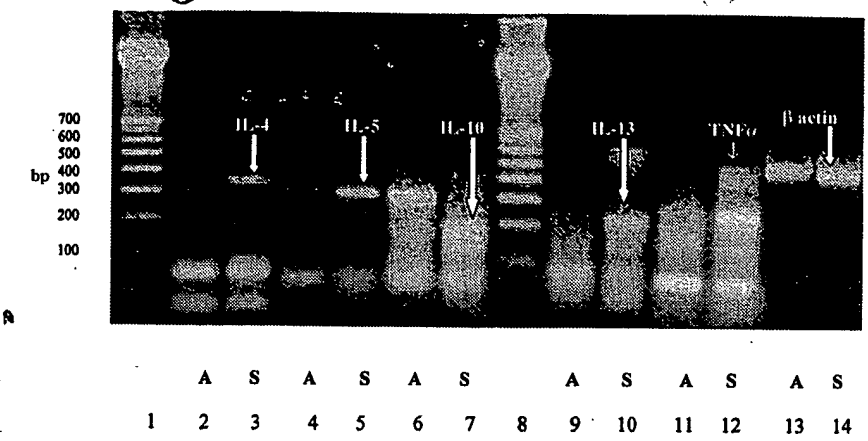

FIGURE 2. Agarose gel showing changes in cytokine/chemokine gene expression in RBL-J41 cells following exposure to clean, filtered Air, and cigarette Smoke. Cells maintained on a collagen substratum at the air/cell culture interface were exposed to Smoke (S) (1:100 dilution) or Air (A) at a rate of one inhalation/min and smoke exposure duration of 16 s per "puff" (*15*). After 10 min, cells growing on collagen treated Transwells were transferred into DMEM and maintained as adhering cells to the collagen substratum for a further 1 h at 37 °C in a standard tissue culture incubator. RNA isolation, RT-PCR, and cDNA synthesis and amplification by PCR were carried out as described in the Experimental Section. The products were analyzed by agarose gel electrophoresis. Lanes 1 and 8 molecular weight standards; lanes 2,4,6,9,11 cytokine expression; and β-actin expression (lane 13) following exposure to Air, lanes 3,5,7,10,12 cytokine and β-actin (lane 14) expression following exposure to Smoke. Despite cloning and gene sequencing it was not possible to identify the band at ~300 bp in lane 6, which is prominent in air exposed cells, but disappeared upon smoke exposure.

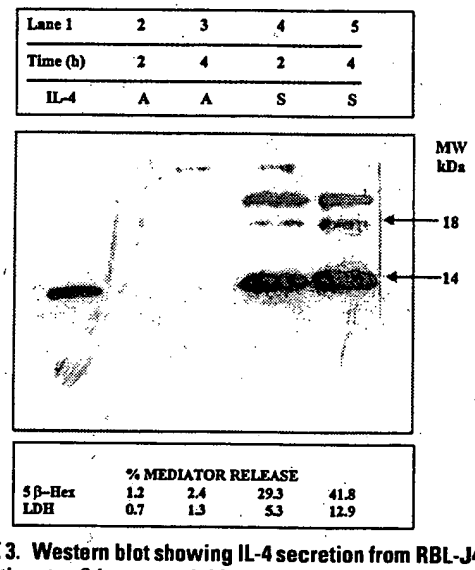

FIGURE 3. Western blot showing IL-4 secretion from RBL-J41 cells in relation to β-hexoseaminidase and LDH release following exposure to Air and Smoke. Secretory responses of RBL-J41 cells maintained in 24 well tissue culture dishes (Costar) covered with 200 μL assay buffer were assessed after 2 and 4 h exposure to cigarette smoke free Air (A) (lanes 2 and 3) and ETS (S) generated by volunteers smoking a commercially available cigarette brand under defined conditions (lanes 4 and 5). Lane 1: positive control for anti-rat IL-4 reactive proteins secreted by CHO cells (*12*). For further experimental details see legend for Figure 1a.

incubator. RNA extraction was carried out on pooled cells from one set of six Transwells following each exposure period, and this provided sufficient RNA for four RT-PCR reactions using a commercial kit (Stratagene 200345). RNA concentration was determined spectrophotometrically. RT-PCR was carried out on 24 μg RNA according to standard procedures. Primers for rat cytokine IL-4 and IL-5 and the house keeping gene β-actin, which was included as an internal standard in all RT-PCR amplifications, have been described previously (*17*). The following primer sequences were designed for rat IL-10: 5′ GCCAAGCCTTGTCAGAAATG, 3′ GTATCCAGAGGGTCTTCAGC; IL-13 5′ TATGGAGCGTGGACCTGACA, 3′ CCTCAGTGGCCATAGCGGAA; and TNFα: 5′ TCCAGAACTCCAGGCGGTGT, 3′ TCTTGATGGCGGAGAGGAGG. cDNA synthesis was performed in one thermal cycle (42 °C for 50 min, 90 °C for 5 min, and 4 °C for 10 min). Following treatment with 1U RNAase H (GIBCO), 0.5 μg cDNA was amplified by PCR over 30 cycles (94 °C for 1 min, 60 °C for 2 min, and 72 °C for 2 min) employing standard procedures (*17*). After electrophoretic separation on 2% agarose gels, separated bands were visualized on an UV transilluminator and compared to a 100 base pair (bp) DNA ladder (Pharmacia). Cytokine synthesis in response to an IgE/DNP mediated stimulus was included for comparison. PCR products were excised from the gel (Wizard PCR, Promega) and ligated into pUC 18 ("Ready to Go", Pharmacia) for sequence analysis of the cDNA products.

Results

Figure 1a shows histamine release from dissociated lung tissue cell preparations enriched in HLMC, and 5-HT or β-Hex from cultured RBL J41 cells, in response to exposure to ETS containing atmosphere under controlled conditions or in a public bar over a period of 4 h. These mediators are specific indicators of mast cell/basophil degranulation (*12–14*). Basal levels of histamine/5-HT/ β-Hex release from control cells kept in a smoke free atmosphere, under otherwise identical condition, were 4/2.5/1.2% of total cellular content of the respective mediator. Basal release was subtracted from the experimental data shown in Figure 1a.

Because the volume of medium covering cells in culture could have an effect on the concentration of smoke derived components interacting with cells, two different volumes of culture medium were used. Cells grown in 24 well tissue culture dishes were covered with 400 μL buffer and exposed to ETS. β-Hex release rose over a 4 h period, to 11.6% above the percentage release from cells exposed to air. A dramatic increase was observed when the intervening fluid layer between cells and atmosphere was reduced by 50% to 200 μL, a volume that just covered the cells. Under these conditions, β-Hex release increased to 42% during the 4 h exposure period to ETS, while LDH release rose to 13%. In contrast, mediator secretion of cells exposed to air remained at basal levels (<3%).

Figure 1b compares β-Hex and LDH release from RBL J41 cells grown on a collagen matrix and maintained at the air/liquid boundary in the exposure chamber with measurements taken over periods of 0, 5, 10, and 15 min exposure to either

Smoke or Air. Direct exposure of cells to freshly generated mainstream smoke produced a large and rapid increase in mediator secretion, which rose to 70% after a 15 min exposure period, while cells exposed directly to Air under identical conditions released 18% β-Hex. LDH release was significantly higher in smoke exposed cells (25% of total LDH) compared to air exposed cells (13% of total LDH).

The effects on the stimulation of cytokine/chemokine transcriptions of a 15 min exposure of RBL J41 cells to filtered Air and Smoke, followed by a 1 h post challenge incubation period prior to RNA extraction is shown in Figure 2. Cells exposed to Air, express low levels of IL-4, 5, 10, and 13 (Figure 2 lanes 2, 4, 6, and 9), like nonactivated cells maintained in a standard tissue culture incubator (data not presented). No transcripts for TNFα were detected in cells exposed to Air (Figure 2, lane 11) or cells grown in a tissue culture incubator (data not shown). Following Smoke exposure, we observed distinct increases in transcript levels of IL-4, IL-5, IL-10, IL-13 (Figure 2 lanes 3, 5, 7, and 10), and *de novo* transcription of TNF-α (Figure 2, lane 12). Transcript levels for β-actin levels were not affected by exposure to Smoke (Figure 2, lanes 13 and 14). Cloning and gene sequencing confirmed the identity ascribed to these bands on the basis of size. Transcription of these cytokines was also observed when RBL cells were triggered via an IgE-mediated antigenic stimulus (L.J.S., Ph.D. Thesis, University of Sheffield 1999).

Our attempts to demonstrate cytokine secretion from Air and Smoke exposed cells were restricted by the lack of antibodies to most of the rat cytokines, whose transcription was induced in Smoke exposed RBL cells, with the exception of IL-4. Figure 3 shows a "Western blot" for IL-4 secretion in relation to β-Hex and LDH release into the supernatant of Air (lanes 2 and 3) and Smoke (lanes 4 and 5) exposed cells.

Discussion

The present study provides clear evidence that mediators of the allergic response are released from cigarette smoke activated cells of mast cell lineage. The slow release of histamine from HLMC and 5-HT/β-Hex from RBL-J41 cells following exposure to a cigarette smoke containing atmosphere, shown in Figure 1a, may reflect cellular responses equivalent to passive smoking. Epidemiological studies and murine models of allergy indicate that "Second hand smoke" (ETS) increases the incidence and severity of allergic and asthmatic responses (*1*–*8*). Mediator secretion was obtained under defined conditions and in a public bar. While there may have been additional air pollutants such as ethanol, furan, or marijuana vapors, which may have caused or contributed to increased mediator release when cells were exposed in a public bar, the differences in mediator secretion, recorded over a period of 4 h between cells exposed to ETS under defined conditions in a room where cigarette smoke was generated by volunteers, and the smoke filled atmosphere of a public house are small and statistically not significant ($p = 0.41$). Therefore we concluded that components in cigarette smoke must account for the cellular responses observed. Furthermore, the dramatic increase in mediator secretion following the reduction in the fluid level above cells indicates that increased accessibility of cells to components in ETS is a critical factor in determining levels of release.

This conclusion is further substantiated by data shown in Figure 1b, which are likely to reflect cellular responses that occur during active smoke inhalation. Mediator release over a 10 min period, assessed as % β-Hex secretion, remains low when cells are exposed to Air (~10%) but rises to ~50% upon exposure to Smoke. An almost 4-fold increase to 70% is observed by 15 min, when compared to Air (18%). Only modest differences in LDH release (5%) are found between smoke containing and smoke free air after 10 min exposure, while β-Hex secretion differs by ~40%. Therefore, cytotoxicity alone cannot explain the dramatic increase in β-Hex release observed in Smoke exposed cells, and it is reasonable to conclude that component(s) in Smoke activate intracellular secretion pathways which culminate in degranulation of mediators of the allergic response.

Since we observed previously increases in IL-4 transcription in both allergen activated RBL and HLMC (*12, 13*), we assessed differences in cytokine/chemokine expression in response to Smoke. Results shown in Figure 2 demonstrate that when cells are exposed to Air, cytokine/chemokine gene expression remains at a basal level. Exposing cells at the air/liquid interface does not induce TNF-α or cytokine gene up-regulation. In contrast, dramatically increased gene expression for IL-4, 5, 10, and 13 and *de novo* synthesis of TNF-α was demonstrated following challenge with Smoke, while transcription levels for the house-keeping gene β-actin remain unchanged. Since few antibodies to rat cytokines are available, we were unable to ascertain secretion levels of most cytokines. To date (see Figure 3) we have only been able to demonstrate, by immunoblotting with an anti-rat IL-4 anti-serum, secretion of IL-4, which is the cytokine essential for the induction of IgE synthesis (*12, 13*) following exposure to ETS, but not Air.

The release of inflammatory mediators and the stimulation of the synthesis of proinflammatory cytokines by cigarette smoke are highly significant in view of the central role of these cytokines in the genesis and pathophysiological responses associated with allergy and allergic asthma (*9, 10*). They provide an explanation for the epidemiological observations that smoking can enhance IgE synthesis. In mice, ETS has adjuvant activity for Th2 responses (*6*), since IL-4 and IL-13 induce Ig class switching from IgM to IgE synthesis in B-cells and promote Th2 development (*6, 9, 10, 12, 13, 17*). IL-5, which is chemotactic for eosinophils and activates their growth and differentiation, can account for the chronic pulmonary eosinophilia associated with smoking (*6*), while IL-10 inhibits Th1 development and stimulates mast cell growth (*4, 7, 8*). TNF-α exerts pleiotropic inflammatory effects and contributes to increased vascular permeability, resulting in edema (reviewed in refs *9*–*11*). The demonstration that IL-13 induces asthma through unidentified mechanism(s), which differ(s) from those implicated in classic IgE-mediated allergic responses (*9, 10*), together with data presented in this study provide an explanation for the result of a recent survey, which showed that ETS is a trigger of asthma attacks in almost 80% of people with asthma (*18*).

The molecular mechanism by which cigarette smoke activates mast cells awaits identification. We have shown in preliminary studies that exposure to Smoke, but not to Air, increases protein tyrosine phosphorylation of a range of RBL proteins (data not shown). The phosphorylation pattern is similar, but not identical, to that observed in response to IgE-mediated degranulation (L.J.S., University of Sheffield, Ph.D. Thesis, 1999).

Active smoking arguably constitutes the largest inhaled oxidant challenge to humans and cellular responses to oxidative stress may lead to mast cell degranulation and proinflammatory cytokine synthesis. Free radicals, oxidants, nicotine, and its derivatives have all been identified in the tar and gas phases of cigarette smoke (*15, 16*). It is particularly relevant to note that oxidative processes can activate transcription factors of the NF-κB family (*19*). In man, there is evidence for a linkage on chromosome 5q31.1 between a gene controlling atopy and the IL-4, -5, and -13 cluster, whose promoter region has an NF-κB recognition sequence (*20*). Furthermore, several components in cigarette smoke are chemically related to particles in diesel exhausts, which have been shown to stimulate ongoing IgE synthesis (*21*), sug-

gesting that related or identical components can account for the IgE adjuvant activity of several environmental pollutants.

Data presented in this study provide evidence for the molecular mechanism(s) underlying the proallergenic and asthma-inducing properties of components in cigarette smoke. Our conclusions are supported by previous epidemiological observations and results of animal experiments (*1*–*10*). The current study has only focused on the effects of cigarette smoke on cells of mast cell lineage. An extension of this work should include other cells of the airways and their mediators, since it is known that oxidants, including ozone, induce release of e.g. cytokines and mitogenic proteases, by alveolar macrophages and airway epithelia (*22*) and stimulate protein tyrosine phosphorylation in T-cells (*23*). Such investigations should lead to an improved understanding of the cellular and molecular mechanism(s) by which environmental pollutants present in cigarette smoke activate the release of proallergenic mediators and influence subsequent adaptive immune responses. The assay system employed in the present study should expedite the identification and possible elimination of components(s) in cigarette smoke responsible for the immune modulatory activity and permit the development/assessment of potential antagonists while reducing the requirement for animal experiments.

Acknowledgments

The collaboration between laboratories in Sheffield and Hannover was supported by a grant from the European Union (EU grant PL931184). We are grateful to Dr. A. Emmendoerfer at the Fraunhofer Institute, Hannover, for the provision of laboratory facilities and Drs. E.M. Carey and M. Wainwright for helpful suggestions during the preparation of the manuscript.

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1. PERSONAL DETAILS

SURNAME: HELM **FORENAMES:** Birgit Anna

DATE OF BIRTH: 04.01.1946 **DEPARTMENT** Mol. Biol. & Biotech.

EDUCATION AFTER SCHOOL:

Norwood Technical College, London		1968-1970
City of London Polytechnic		1970-1972
Bedford College, Univ. of London	(undergraduate)	1972-1975
ibid	(postgraduate)	1976-1978

QUALIFICATIONS:

O.N.C.	1970
H.N.C.	1972
B.SC. (Biochemistry, Class 1)	1975
Ph.D. (Biochemistry)	1980

MEMBERSHIP OF LEARNED SOCIETIES:

Biochemical Society
British Society for Immunology
International Society for Molecular Recognition

CURRENT APPOINTMENTS:

Lecturer, Dept. Molecular Biology & Biotechnology University of Sheffield, S10 2TN	1989
Senior Lecturer, ibid	1999

PREVIOUS APPOINTMENTS

Research Associate Department of Biophysics, King's College, London	1983-1988
Research Associate Texas A & M University, College Station, Texas, USA	1982
Lecturer (temporary) Bedford College, University of London	1978-1981
Medical Laboratory Technician University College, London	1968-1972

2. RESEARCH

- **(1) Structure/function relationship in human immunoglobulin E and its receptors**
- **(2) Proteomics applied to signal transduction in cells of the immune system**
- **(3) Design of rational therapeutic interventions in allergic and parasitic disease**
- **(4) Development of biological assay systems to monitor potential allergenicity of environmental allergens and pollutants**

3. RESEARCH GRANTS AND CONTRACTS

Since my appointment to a lectureship in Sheffield in 1989, I have raised over £1.9 million (£1.75 million personnally) for research projects concerned with the design of rational therapeutic interventions in allergic and asthmatic disease.

Funding obtained during the 2000/2001 session:

- **A proteomics based approach to the definition of IgE-receptor mediated signalling pathways in mast cells**

Sponsor	BBSRC
Dates:	1/1/2001-31/12/2003
Value	£ 259,864

Assessing the molecular basis of allergenicity associated with cornstarch and latex

Sponsor	BBSRC/SSL International
Dates	1/10/2001-30/9/2004
Value	£ 73, 000

Research funds raised between 1989 and 2000:

- **A dog model of Blocking IgE:FcεRI interaction**

Sponsor	Allergy Therapeutics
Dates:	1/10/99-30/9/2002
Value	£ 126,000

- **Licensing fee (non-exclusive) for genetically engineered mast cell line**

Sponsor	Peptide Therapeutics
Dates:	6/99-5/2000
Value	£ 12,500

- **Structure/function relationship in human IgE and its receptors**

Dates	10/98-9/2001
Funding body	BBSRC/CASE with Eclagen/Biovation
Value	£50,000

- **Structure based design and synthesis of antagonists of the allergic response**

Dates:	10/98-7/2001
Funding body	Wellcome Trust
Principal investigators	Spivey, A.C. & B.A. Helm
Grant value	£ 49,073; supplementary support to

- **Structure based design and synthesis of antagonists of the allergic response**

Dates:	11/97-10/2000
Funding body	Wellcome Trust
Principal investigators	Spivey, A.C. & B.A. Helm
Grant value	£ 147,812

- **Protein Engineering of the high-affinity receptor complex for immunoglobulin E**

Dates:	10/97-9/2000
Funding body	National Asthma Campaign
Principal Investigator	B.A. Helm
Grant value	£126,091

- **Structure-function studies on human IgE and its receptors (renewal)**

Dates	6/97-5/99
Funding body	NATO
Principal investigators	B.A. Helm & E.A. Padlan (NIH, Bethesda, USA)
Grant value	£ 4000

- **Large scale expression of human IgE-Fc and receptor fragments for structure determination**

Dates:	5/97-4/98
Funding body	MRC
Principal Investigator	B.A. Helm
Grant value	£ 39,184

- **An investigation into regulatory circuits in allergic disease**

Dates	10/94-3/98
Funding body	European Community
Principal Investigator	B.A. Helm
Grant value	ECU 150,000

Molecular basis of the allergenicity of bee venom phospholipase A_2

Dates	3/96-2/97
Funding body	British Council
Grant value	£ 2000

- **Development of an assay system for the screening of potential allergenicity**

Dates:	10/95-9/98
Funding body	Health & Safety Executive
Principal investigator	B.A. Helm
Grant value	£ 21,600

- **Structure-function studies on human IgE and its receptors**

Dates	6/95-5/97
Funding body	NATO
Principal investigators	B.A. Helm & E.A. Padlan (NIH)
Grant value	£ 4000

- **Signal transduction in cells of mast cell lineage**

Dates	10/94-9/97
Funding body	GLAXO
Principal Investigator	B.A. Helm
Grant value	£ 49,500

- **Expression of the ligand binding domain of human FcεRI in *Pichia pastoris***

Dates	10/93-9/96
Funding body	Pfizer U.K.
Principal Investigator	B.A. Helm
Grant value	£ 30,000

- **A study of gene expression in releasing and non-releasing variants of basophilic cell line**

Dates	10/93-9/96
Funding body	National Asthma Campaign.
Principal Investigator	B.A. Helm
Grant value	£ 102,202

- **Expression of components of the IgE/receptor/effector system for structure/function determination**

Dates	1/91-12/94
Funding body	SERC/DTI/EURO DPC (LINK)
Principal Investigator	B.A. Helm
Grant value	£ 417,560

- **Recognition of class-specific Fc-receptors by human IgE**

Dates	7/89-6/92
Funding body	MRC/ EURO DPC
Principal Investigator	B.A. Helm
Grant value	£ 216,865

4. TEACHING

4.1 CURRENT UNDERGRADUATE TEACHING

Undergraduate teaching involves lecturing (six lectures and one tutorial) to ~120 second year students on a course entitled "Gene Expression and Regulation", nine lectures and two tutorials to ~120 second year students on a course entitled "Biochemical Messengers", six lectures to ~70 third year students on a course in "Molecular Immunology". I also supervise three laboratory classes for ~180 first year students and laboratory research projects for three third year undergraduate students. I am tutor to four first and five second, and four third year undergraduate students and hold academic tutorials (one per months) to second year undergraduate students.

4.2 PREVIOUS UNDERGRADUATE TEACHING

There has been a continuous increase in my undergraduate and postgraduate teaching load during the past five years.

4.3 RESEARCH SUPERVISION

Since 1990, I have supervised nineteen post-graduate research students (sixteen full-time and three part-time). Currently, I supervise seven postgraduate students, one post-doctoral research associate, one research assistant and one technician. There is a monthly one-hour journal club for postgraduate research students and weekly laboratory meetings. Twelve students have been awarded the degree of Ph.D. All full-time home students I supervised completed their theses in less than four years and no re-submissions were required. Two of my research students received prizes for work carried out under my supervision.

4.4 TEACHING INNOVATION AND DEVELOPMENT

In collaboration with my former research associate, N. Rhodes, we developed a computer based teaching and learning package on allergy to complement lectures in the third year undergraduate Molecular Immunology Course. I enrolled in a M.Ed. Course offered by the University, which led to the implementation innovative teaching practices designed to address the challenge presented by teaching of large groups.

5. ADMINISTRATION

As course co-ordinator for three third year modules, I am responsible for the co-ordination of the Molecular Immunology module, and the allocation of both laboratory and library research projects to 126 undergraduate students for supervision by some thirty five members of staff. With 40 credits, these modules represent the core of final year teaching. I am a member of the departmental biochemistry teaching group, and a group concerned with improvements in teaching quality. In addition, I am a member of three post-graduate committees monitoring the progress of research students, act as examiner in connection with the award of Ph.D. theses. I have organised postgraduate courses with invited external lectures, supported by funds I raised from industrial sponsors. I am Departmental representative for contact with 'Year Abroad' students (1993 – present).

6. PROFESSIONAL ACTIVITIES

I have presented papers and chaired plenary sessions at several national and international meetings in Britain, Germany, Switzerland, Poland, USA, Canada, Japan and India. I also presented papers at major industrial and academic institutions in this country and overseas. In recent years, research originating from my laboratory has received considerable coverage in the national and international press.

I referee grant applications on behalf of the European Union (Expert Evaluator, Framework 5), the MRC, BBSRC, the Wellcome Trust and the National Asthma Campaign. I review manuscripts for Immunology Today, International Archives of Allergy, the European Journal of Immunology, the Scandinavian Journal of Immunology, and the European Journal of Allergy and Clinical Immunology and I am a co-editor of "Current Opinion in Biotechnology". I am a consultant to Allergy Therapeutics and Scholl Seaton International, and a member of the Global Advisory Panel to Regent Medical to advise the companies on allergy related research.

7. PUBLICATIONS

I am an author/co-author on more than 40 research papers, most of which were published in refereed international journals, including Nature, Proc. Natl. Acad. Sci., J. Immunol., J. Biol. Chem., Eur. J. Immunol., which have an impact factor of 6 or above, and a co-inventor of six patents.

7.1 Publications in refereed journals and conference proceedings

P = principal author J = joint author I.F. = impact factor of journal N/A not available

Manuscripts submitted/in preparation:

1. I. Sayers, Housden, J.E., Spivey, A.C.,. and B.A. Helm (2000) Identification of a single residue in human IgE critical for FcεRII/CD23 interaction*: J. Biol. Chem.*

2. Helm, B. Housden J., Good, S. Carrol, K., A.C. Spivey and Carey, E. (2001) Identification of novel targets for therapeutic interventions in allergy and asthma. In *Recent Advances in Molecular Biology and Immunology*. pp 65-80. Eds. C N Ramchand, P N Nair & B Pilo. Allied Publs. Ltd. New Delhi.

Manuscripts published/ in press:

1. Carroll, K., Carey, E and Helm, B. (2002) Protein mapping in rat basophilic leukaemia cells. J. Chromatography, in press.

2. Carroll, K., Ray, K., Helm, B and Carey, E. (2001) Differential expression of Rab3 isoforms correlates with variations in the secretory phenotype of mast cells. *Eur. J. Cell Biol.* 80, 295-302.

3. Carroll, K., Ray, K., Helm, B. and Carey E. (2000) Two-dimensional electrophoresis reveals differential protein expression in high-and low-secreting variants of the rat basophilic leukaemia cell line. *Electrophoresis* **21**, 2476-86.

4. Smyth, L.J., Machado, D.C., Good, S., Upton, A., Aufderheide M., and Helm, B.A. (2000) Assessment of the molecular basis of pro-allergenic effects of cigarette smoke. *Environmental Science and Technology*, **34,** 1370-1374. (P/I.F. 3.5)

5. Sayers, I. and B.A. Helm (1999) Structural basis of human IgE-Fc receptor interactions. *Clin Exp Allergy* **29**: 585-94 (P/I.F. 3.8)

6. I. Sayers, S.A. Cain, J.R.M. Swan, M.A. Pickett, P.J. Watt, S.A. Holgate, E.A. Padlan, P. Schuck and B.A. Helm (1998) Amino acid residues that influence FcεRI mediated effector functions of human immunoglobulin E. *Biochemistry* **37**, 16152-16164, (P/ I.F.4.82).

7. B.A. Helm, I. Sayers, S.A. Cain, J.R.M. Swan, L.J. Smyth, M. Suter, D.C. Machado. A.C. Spivey, E.A. Padlan (1998) Protein and cell engineering of components of the human immunoglobulin E receptor/effector system: applications for therapy and diagnosis. *Technology and Health Care* **6**, 195-207, (P/ I.F. 1.6).

8. B.A. Helm, I. Sayers, E.A. Padlan, J.E. McKendrick, and A.C. Spivey (1998) Structure/function studies on IgE as a basis for the development of rational IgE antagonists. *Allergy* **53**, 85-90, (P/ I.F.2.0).

9. Helm, B.A, B.A., Spivey, A.C. and Padlan, E.A. (1997) Peptide blocking of IgE/receptor interaction: Possibilities and pitfalls. *Allergy* **52**:6-13, (P/ I.F.2.0).

10. Machado, D.C., Horton, D., Harrop, R., Peachell, P.T. and Helm, B.A. (1996) Potential Allergens stimulate mediator release and interleukin-4 synthesis from cells of mast cell lineage in the absence of sensitisation with antigen-specific IgE. *Eur. J. Immunol.* **26**:2972-2980, (P/ I.F.6.0).

11. Helm, B.A., Sayers, I., Higginbottam, A., Machado, D.C., Ling, Y., Ahmad, K., Padlan, E.A. and Wilson, A.P.M. (1996) Identification of the mast cell binding region in human IgE. *J. Biol. Chem.* **271**:7494-7500, (P/ I.F.7.4).

12. Dudler, T., Machado, D.C., Kolbe, L., Annand, R, Rhodes, N., Gelb, M., Koelsch, E., Suter, M. and Helm, B.A. (1995) A link between catalytic activity, IgE-independent mast cell activation and allergenicity of bee venom phospholipase A 2. *J. Immunol.* **155**: 2505-2513. (P/I.F. 7.6).

13. Bingham, B.R., Monk, P.N. and Helm, B.A. (1994) Defective protein phosphorylation and Ca++ mobilisation in a low secreting variant of the rat basophilic leukaemia cell line. *J. Biol. Chem.* **269**:19300-19306, (P/7.4).

14. Helm, B.A. (1994) Is there a link between the nature of agents that trigger mast cells and the induction of IgE synthesis? *Adv. Exp. Med.* **347**: 1-10. (I.F. N.A)

15. Padlan, E.A. and Helm, B.A. (1993) Modeling of the lectin homology domain of the human and murine low-affinity FcεRII/CD23. *Receptor* **3**:325-341. (J/I.F. 2.0)

16. Monk, P.N., Bingham, B.R., Ahmad, T.B. and Helm, B.A. (1993) Characterisation of defective phorbol ester responses in a low secreting rat basophilic leukaemia cell variant. *Receptor* **3**: 77-86, (J/I.F. 2.0)

17. Wilson, A.P.M., Machado, D.C., Rhodes, N., Ahmad, T., Pullar, C. and Helm, B.A.(1993) Revisiting the Basophil degranulation test. *J. Clin. Immunoassay* **16**: 91-95, (J/I.F. 2.0)

18. Pullar, C.E., Wilson, A.P.M. and Helm, B.A. (1993) Expression of the α–chain of the human high affinity receptor for IgE in a cell line capable of mediating stimulus secretion coupling. *J. Immunol.* **150**:1276, (J/I.F. 7.6)

19. Bingham, B.R., Ahmad, T., Monk, P.N. and Helm, B.A. (1993) Impaired mediator secretion and high
affinity receptor phosphorylation in a variant of the rat basophilic leukaemia cell line. *J. Immunol.* **1 50**: 1266. (J/I.F.7.6)

20. Helm, B.A., Moreira Machado, D. C. and N. Rhodes (1993) A link between the nature of mast cell triggering agents and the induction of an IgE response. *J. Immunol.* **150**: 1014, (P/I.F.7.6)

21. Padlan, E.A. and Helm, B.A. (1993) A modelling study of IgE and IgE/receptor interaction. *Biochem. Soc. Transactions* **27**: 963-969 (J/ I.F. 0.6)

22. Wilson, APM, Pullar, CE, Camp, A, Helm, BA (1993) Human IgE mediates stimulus secretion coupling in rat basophilic leukaemia cells transfected with the a-chain of the human high-affinity receptor. Eur. J. Immunol. 23: 240-244 (P/I.F. 6.0)

23. Helm, B.A. and Padlan, E.A. (1993) Allergens, Immunoglobulin E, and the Quest for a Rational Inhibitor of the allergic response. In Molecular Biology and Immunology of Allergens pp 91-100. Eds. Kraft, D. and Sehon, A.CRC Press Inc. (P/I.F. NA)

24. Padlan, E.A. and Helm, B.A. (1992) A modeling study of the α–subunit of the human high-affinity receptor for immunoglobulin E. *Receptor* **2**:129-144, (J/I.F. 2.0)

25. Helm, B.A., Ling, Y., Rhodes, N., and Padlan, E.A. (1992) Can structural and functional studies on components on the IgE/receptor/effector system assist the design of rational IgE antagonists? In *Progress in Allergy and Clinical Immunology* **2**, pp 589-599. Eds. Miyamato, T. and Okuda, M. Hogrefe & Huber Publs. Bern, (P/I.F. N.A)

26. Helm, B.A., Ling, Y., Teale, C., Padlan, E. and Brueggemann M (1991) The nature and importance of the inter-ε-chain disulphide bond in human IgE. *Eur. J. Immunol.* **21**: 1543-1548, (P/ I.F. 6.0)

This publication was chosen by the editor of Allergy as "outstanding in its field" and has been reprinted in an abbreviated version:

26. Helm, B.A., Ling, Y., Teale, C., Padlan, E. and Brueggemann M (1992) The nature and importance of the inter-ε-chain disulphide bond in human IgE. *Allergy Digest* **9**:31-32.

27. Helm, B.A. (1989) The Interaction of human IgE with Class-specific Fc-receptors. In *Advances in the Biosciences* **74**: 83-90. Eds. Merret T. and El Shami, A.S. 1989 Pergamon Press. (P/ I.F. NA)

28. Helm, B.A., Short, N. and Geha, R.S. (1989) The mast cell binding site in human IgE: A single ε–chain can engage the high-affinity receptor. In *Progress in Allergy and Clinical Immunology* **1**, pp 96-100. Eds. Pichler et al. Hogrefe & Huber Publs. Bern. (P/ I.F. NA)

29. Vercelli, D., Helm, B.A. Marsh, P.J., Padlan, E.A., Gould, H.J. and Geha, R.S. (1989) The IgE binding site for low-affinity Fc-receptors.(1989) In *Progress in Allergy and Clinical Immunology* **1**, pp101-106. Eds. Pichler et al. Hogrefe & Huber Publs. Bern, (J/ I.F. NA)

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7.2 Other Publications

Co-editor with E.A. Padlan of *Current Opinion in Biotechnology* **8** No 4, 1997.

P Editorial overview: Birgit A. Helm and Eduardo A. Padlan. Protein Engineering: From plants to animals, from big to small, from outside to inside and other advances. *Current Opinion in Biotechnology* 1997, **8:**397-399.

7.3 Book Chapters

P 1. Regulation of IgE synthesis
Helm, B.A. In *Atlas of Asthma*. Eds. Holgate S. and R. Djukanovic, 1999 pp 44-47. Parthenon Publishing.

J. 2. Development of Structural Models For a Study of the Interaction of Human IgE with Class-specific Fc-receptors.
Eduardo A. Padlan and Birgit A. Helm. In *Allergic Mechanisms and Immunotherapeutic* Strategies pp 1-15. Ed. A.M. Roberts and M.R. Walker. 1997 John Wiley and Sons Ltd.

P 3. Protein Engineering of IgE antibodies: Potential applications.
Helm, B.A., Ling, Y., Mackie, S., and Padlan, E.A. In *Molecular Diagnostics: Research towards applications* pp 312-332. Ed. A.M. Roberts and M.R. Walker. 1993 Blackwell Scientific Publications.

J 4. IgE in allergic inflammation
Marsh, P., Helm, B.A., and Gould, H.J. In *Pharmacia Allergy Research Foundation*, pp 26-31. Ed. S.G.O. Johannson 1988.

J. 5. Transfer of maternal proteins to a developing embryo
Cheesman, D.F. and Helm, B.A. In *Biophysical Information Transfer* pp 637-642. Eds. Vassileva Popova, J.G. and Jensen, E.V. 1979. Plenum Press N.Y.

OTHER INNOVATIVE AND CREATIVE ACTIVITIES

8.1. PATENTS

1. Polypeptide competitor for human IgE (with Prof. H.J. Gould.)
International patent 899604292.

2. Low-affinity IgE competitor (with Prof. H.J. Gould & Dr. P.B. Marsh)
International patent 87904292.

3. Improvements relating to allergen testing and diagnosis (with Drs. P.Wilson, D. C. Machado, C.E. Pullar, and A. Camp)
International patent GB/93/02430.

4. Inhibition of IgE-mediated allergies by a human IgE-derived oligopeptide (with Dr. E.A. Padlan)
USA Provisional Patent Application 29768, filed Dec. 1996.

5. Applications of IgE variant Gly352
Provisional Patent Application, filed Oct. 1998

6. Applications of IgE variant Gly333
Provisional Patent Application, filed Oct. 1998

9. Research in progress and future perspectives

Allergic diseases, characterised by an increase in levels of antibodies of the IgE isotype, affect some 25% of the population in industrialised countries, and more than 10% of children suffer from IgE-mediated asthma. Currently, there are no satisfactory therapeutic interventions and the disorder has a profound socio-economic impact. The cost of disease management in Britain is in excess of £ 1 billion p. a. There is ample evidence that the molecular mechanisms which stimulate immune responses to parasitic infestations (another economically important disease) are also linked to the induction of IgE responses. The overall aim of my research programme is to generate information to assist the development of rational therapeutic interventions in allergic and parasitic disease.

Research is currently in progress

- to identify the complementary site of interaction between IgE and its high and low affinity receptors. As a result of our identification of the high-affinity receptor-binding region in IgE, we have designed a "lead compound" (patent filed with E.A. Padlan, NIH, Bethesda, USA) which blocks IgE/receptor interaction. The further development of this lead compound (in collaboration with Dr. A.C. Spivey, Dept. Chemistry) will benefit from structure/function studies on the extracellular domains of the high- and low-affinity receptors for IgE, which we have over-expressed (in collaboration with Dr. M. Attwood, MBB). Structure studies by nmr (in collaboration with Dr. J. Waltho, MBB) and crystallisation trials have been initiated;

- to develop an allergic human SCID mouse model system for the *in vivo* assessment of anti-allergic and anti–parasitic drugs; which we are developing in collaboration with Dr. A.C. Spivey, (Dept. Chemistry, Sheffield) and Prof. A. Wilson, Univ. York);

- to establish a biological assay system to assess the potential allergenicity aero-allergens and environmental pollutants with collaborators at the Fraunhofer Institute in Hannover, Germany (Prof. M. Aufderheide) and colleagues at ScHARR and the Health and Safety Laboratory, Sheffield (Prof. T. Higenbottam). Such an assay system has important applications in clinical and occupational medicine.

- to apply a functional proteomics approach to the study of immunoglobulin-mediated signal transduction in cells of the immune system (with Prof. M. Gelb, University of Seattle).